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Détermination de la Masse et des Dimensions des Protéines en Solution par la Diffusion Centrale des Rayons X Mesurée à l'Échelle Absolue : Exemple du Lysozyme

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A technique is described whereby small-angle X-ray scattering experiments are performed on an absolute scale, i.e. the intensity of the diffracted beam is compared to the energy of the primary beam. A method of computation is given whereby the experimental measurements are first transformed into a normalized function. This leads to the determination of several parameters which are related to the structure of the sample. In the study of dilute solutions of monodisperse globular particles the molecular weight, radius of gyration, volume, external surface, and internal solvation of the particles can be determined. Some of these parameters (volume, surface and solvation) can in fact be determined only if the electron density is assumed to be uniform inside the particle: the validity of this assumption is discussed. The experimental values of some of these parameters can be interpreted in terms of a simple geometric form. The case is considered in which the particle is an ellipsoid of revolution: different combinations of the experimental parameters are given which can be used to determine the dimensions of the ellipsoid.

Dilute solutions (1 to 5%) of lysozyme in water have been studied with this technique. The molecular weight is found to be 14,100, in close agreement with other techniques. The shape of the molecule is shown to be satisfactorily described by an ellipsoid of revolution. The intrinsic viscosity and the diffusion coefficient agree with the values calculated from the dimensions of the ellipsoid given by the X-ray measurements.

1. Introduction

Nous avons mis au point récemment (Luzzati 1960, abrégé L. dans la suite) une technique de diffusion centrale des rayons X où l'intensité du rayonnement diffusé est comparée à l'énergie du faisceau incident, ce qui permet d'effectuer des mesures absolues.

Cette technique nous a permis d'entreprendre l'étude de la structure, en solution, de molécules d'intérêt biologique: un premier exemple est celui de l'acide désoxyribonucléique (Luzzati, Nicolaieff & Masson, 1961).

Avant de présenter d'autres résultats il nous a paru utile de consacrer un bref mémoire à la technique expérimentale et de calcul, pour en discuter les caractéristiques essentielles, et pour envisager les différentes applications pratiques. Nous étayerons ces considérations générales sur l'exemple du lysozyme, protéine bien caractérisée par d'autres méthodes.

2. Technique Experimentale et de Calcul

(a) *L'appareil*

Le faisceau de rayons X, issu d'un tube à foyer fin dont l'émission est stable, est focalisé sur un segment de droite, parallèle à la fente d'entrée d'un compteur de Geiger, au moyen d'un monochromateur à lame de quartz courbée, qui isole la raie $K\alpha_1$, du cuivre. L'échantillon, enfermé dans une cuve plane, à fenêtres de mica, est centré sur l'axe de rotation d'un goniomètre, dont le bras tournant porte le compteur de Geiger. Un jeu de fentes linéaires définit et limite la largeur du faisceau incident: la longueur est au contraire très grande, de manière à ce que le système de collimation puisse être traîté comme s'il était linéaire et infini. Un ensemble de filtres étalonnés permet d'atténuer le faisceau de rayons X dans des rapports qui atteignent 10^6, et ceci avec une bonne précision.

Dans chaque expérience on enregistre d'une part, la distribution angulaire de l'intensité diffusée par l'échantillon, d'autre part l'énergie du faisceau incident (convenablement atténué par des filtres), pendant la rotation du goniomètre; la vitesse peut être aussi faible que 1°. 2θ en 8 heures. On enregistre ensuite, dans les mêmes conditions, l'intensité diffusée par la même cuve, remplie de solvant.

L'épaisseur de la cuve est mesurée à l'aide d'un double microscope.

(b) *Les calculs*†

On mesure, dans chaque expérience, l'intensité $I(s)$ diffusée en fonction de s, l'énergie totale du faisceau incident $\int i_0(s)\mathrm{d}s$, l'épaisseur de l'échantillon (définie par le nombre η d'électrons par cm^2): ces mesures sont effectuées d'une part avec la solution, d'autre part avec le solvant.

On dégage ensuite les résultats des contingences expérimentales, en calculant une fonction normée (ν est une constante égale à $\lambda^2.7{,}9\ 10^{-26}$ c.g.s.)

$$j_n(s) = \left[\frac{I(s)}{\eta\nu\int i_0(s)\mathrm{d}s}\right]_{\text{solution}} - \left[\frac{I(s)}{\eta\nu\int i_0(s)\mathrm{d}s}\right]_{\text{solvant}} \tag{1}$$

qui, dans les conditions idéales d'une collimation linéaire infinie, ne dépend que de la structure de l'échantillon (voir L.).

Si on suppose que les particules sont toutes identiques, et que leur forme est globulaire (plus exactement qu'aucune des dimensions n'est grande devant l'inverse de la plus petite valeur de s), on peut déterminer leur masse et leur rayon de giration R_0. Pour le faire il convient de décomposer $j_n(s)$ en la somme de la fonction de Gauss osculatrice à l'origine et d'une fonction $\phi(s)$.

$$j_n(s) = j_n(0)\exp\left(-\frac{4\pi^2}{3}R\ s^2\right) + \phi(s) \tag{2}$$

† La notation est ici celle des mémoires précédents (Luzzati, 1960; Luzzati *et al.*, 1961): rappelons la définition des paramètres le plus souvent employés.

s	est le paramètre qui définit l'angle de diffusion: $s = 2\sin\theta/\lambda$ (Å^{-1})
ρ_0	est la densité électronique du solvant, qu'on admet constante (él. Å^{-3})
ρ_1	est la densité électronique moyenne à l'intérieur des particules de soluté, gonflées de solvant (él. Å^{-3})
v (ou ψ)	est le volume spécifique partiel (ou le volume spécifique partiel électronique) du soluté dans le solvant ($\text{cm}^3\ \text{g}^{-1}$ ou Å él.$^{-1}$)
c (ou c_e)	est la concentration, mesurée par le rapport entre la masse (ou le nombre d'électrons) du soluté et la masse (ou le nombre d'électrons) de la solution
M (ou m)	est la masse molaire (ou le nombre d'électrons) d'une molécule de soluté
v_1	est le volume d'une molécule de soluté, gonflée de solvant (Å^3)
S_1	est l'étendue de la surface extérieure d'une molécule de soluté, gonflée de solvant (Å^2)

Si les effets de corrélation en position et orientation entre les particules de soluté, sont négligeables, la masse et le rayon de giration ont pour expression (L., 26, 28, 29):

$$m = c_e (1 - \rho_0\psi)^2 i_n (0) \tag{3}$$

$$i_n(0) = 2\sqrt{\frac{\pi}{3}}\, j_n(0) R_a - \frac{1}{\pi}\int_0^\infty s^{-2}\phi(s)\mathrm{d}s \tag{4}$$

$$R_0^2 = \frac{R_a^2 + \left[\frac{9\sqrt{3\pi}}{16\pi^4}\int_0^\infty s^{-4}\phi(s)\mathrm{d}s\right] \times [j_n(0) \times R_a]^{-1}}{1 - \left[\frac{\sqrt{3\pi}}{2\pi^2}\int_0^\infty s^{-2}\phi(s)\mathrm{d}s\right] \times [j_n(0) \times R_a]^{-1}} \tag{5}$$

Dans la pratique on élimine les effets des corrélations en déterminant les valeurs de m, R_a et R_0 à différentes concentrations à l'aide des équations (2), (3), (4) et (5), et en les extrapolant vers $c = 0$.

On peut déterminer les valeurs de $j_n(0)$ et de R_a en utilisant la représentation $\log j_n(s)$ en fonction de s^2 proposée par Guinier (1939): les premiers points s'alignent sur une droite, dont la pente est proportionnelle à R_a^2, et dont l'ordonnée à l'origine est égale à $j_n(0)$. En soustrayant de $j_n(s)$ la fonction de Gauss (voir 2) on obtient $\phi(s)$, et on peut calculer tous les termes des équations (4) et (5). Par ailleurs le paramètre R_a est en soi intéressant, car on connaît sa définition géométrique (Luzzati, 1957), qui ne dépend que de la structure des particules du soluté.

Jusqu'ici on n'a pas fait d'hypothèses sur la structure interne des particules. Un modèle particulièrement fécond est celui où la densité électronique est uniforme au sein du soluté: bien que cette condition ne puisse être respectée que de manière approchée, nous avons montré dans l'Appendice qu'on peut éliminer l'effet des fluctuations à courte distance de la densité électronique, en soustrayant de $j_n(s)$ une constante δ^*, qui est en général très petite devant $j_n(0)$. Dans ce cas la condition nécessaire pour que ce modèle soit valable est que les points $s^3 j_n(s)$, représentés en fonction de s^3, s'alignent pour s grand, sur une droite; la pente de cette droite est δ^*; l'ordonnée à l'origine est (L., 22 et 27):

$$\frac{1}{16\pi^2}\frac{S_1}{v_1}(\rho_1 - \rho_0)c_e(1 - \rho_0\psi) \tag{6}$$

En interprétant les données expérimentales selon ce modèle il est possible de déterminer plusieurs paramètres: la différence $(\rho_1 - \rho_0)$ entre la densité électronique moyenne à l'intérieur du soluté et la densité électronique du solvant, le volume v_1 de chaque particule et le rapport S_1/v_1 entre surface extérieure et volume

$$i^*(s) = j_n(s) - \delta^* \tag{7}$$

$$\int_0^\infty 2\pi s j_n^*(s)\mathrm{d}s = c_e(1 - \rho_0\psi)\left\{\rho_1 - \frac{\rho_0}{1 - c_e(1 - \rho_0\psi)}\right\} \tag{8}$$

$$\frac{1}{16\pi^2}\frac{\int_0^\infty 2\pi s j_n^*(s)\mathrm{d}s}{\lim\limits_{s\to\infty} s^3 j_n^*(s)} = \frac{v_1}{S_1}\frac{1}{\rho_1 - \rho_0}\left\{\rho_1 - \frac{\rho_0}{1 - c_e(1 - \rho_0\psi)}\right\} \tag{9}$$

$$v_1 = \frac{i_n(0)}{\int_0^\infty 2\pi s j_n^*(s)\mathrm{d}s} \tag{10}$$

Lorsque la concentration c_e n'est pas trop élevée les équations (8) et (9) se simplifient:

$$\rho_1 - \rho_0 \simeq \frac{\int_0^\infty 2\pi s j_n^*(s)\mathrm{d}s}{c_e(1-\rho_0\psi)} + \rho_0 c_e(1-\rho_0\psi) \tag{11}$$

$$\frac{S_1}{v_1} \simeq \frac{16\pi^2 \lim_{s\to\infty} s^3 j_n^*(s)}{\int_0^\infty 2\pi s j_n^*(s)\mathrm{d}s}\left[\rho_1 - \frac{\rho_0 c_e(1-\rho_0\psi)}{\rho_1-\rho_0}\right] \tag{12}$$

Les équations (8) et (9) sont valables à toute concentration, et même dans le cas où les particules ne sont pas toutes identiques, pourvu que la densité électronique moyenne ρ_1 soit partout la même: l'expression de v_1 (10), au contraire, n'est valable que lorsque le système est monodisperse, et après extrapolation à $c = 0$.

La valeur de ρ_1 permet de déterminer en outre le taux de solvatation interne de la particule en solution, car ρ_1 se réfère aux particules gonflées de solvant. Définissons ce taux par le rapport α entre les électrons de l'eau de solvatation et ceux de la particule anhydre. Si on admet que les volumes spécifiques partiels de solvant et soluté sont indépendants on a:

$$\frac{m(1+\alpha)}{\rho_1} = m\psi + \frac{\alpha m}{\rho_0} \tag{13}$$

d'où on obtient:

$$\alpha = \rho_0\frac{1-\rho_1\psi}{\rho_1-\rho_0} = \rho_0\left[\frac{1-\rho_0\psi}{\rho_1-\rho_0} - \psi\right] \tag{14}$$

(c) *Les dimensions de l'ellipsoïde équivalent*

Les expériences de diffusion centrale des rayons X ne permettent pas de déterminer la configuration des molécules de soluté de manière précise et univoque. La coutume, introduite par les hydrodynamiciens (Sadron, 1942, 1953), veut qu'on assimile la forme extérieure de chaque particule à un ellipsoïde de révolution, dont le comportement dans les expériences qu'on cherche à interpréter, est "équivalent" de celui de la particule; il suffit alors de comparer les valeurs expérimentales des différents paramètres avec les valeurs théorique relatives aux ellipsoïdes de révolution, pour déterminer la longueur des axes.

La détermination de la forme et des dimensions de l'ellipsoïde peut être facilitée par l'emploi d'abaques. Soient a, b et b les longueurs des trois demi-axes, soit $p = a/b$ l'allongement: on connaît les expression de v_1, R_0 et S_1:

$$v_1 = \frac{4\pi}{3}pb^3 \tag{15}$$

$$R_0 = \sqrt{\frac{2+p^2}{5}}\,b \tag{16}$$

$$S_1 = \begin{cases} 2\pi b^2\left[1 + \dfrac{p^2}{\sqrt{p^2-1}}\ \text{arc sin}\ \dfrac{\sqrt{p^2-1}}{p}\right] & p > 1 \\ 2\pi b^2\left[1 + \dfrac{p^2}{\sqrt{1-p^2}}\ \text{arc th}\ \sqrt{1-p^2}\right] & p < 1 \end{cases} \tag{17}$$

On peut combiner ces trois paramètres de manière à obtenir des fonctions qui dépendent seulement de l'allongement p: nous utilisons ici $\dfrac{3v_1}{4\pi R_0^3}$ et $R_0\dfrac{S_1}{v_1}$, que nous avons re-

présentés en fonction de p dans Fig. 1. Il peut être parfois plus commode d'utiliser R_a, qu'on obtient de manière plus directe que R_0: nous avons tracé également dans Fig. 1 les fonctions $3v_1/4\pi R_a^3$ et $R_a S_1/v_1$. (Les rapports R_a/R_0 en fonction de p, ont été déterminés par Guinier & Fournet, 1947).

Il faut remarquer toutefois que l'ellipsoïde dont le rayon de giration et le volume sont ceux de la particule n'a pas nécessairement la même surface extérieure que cette

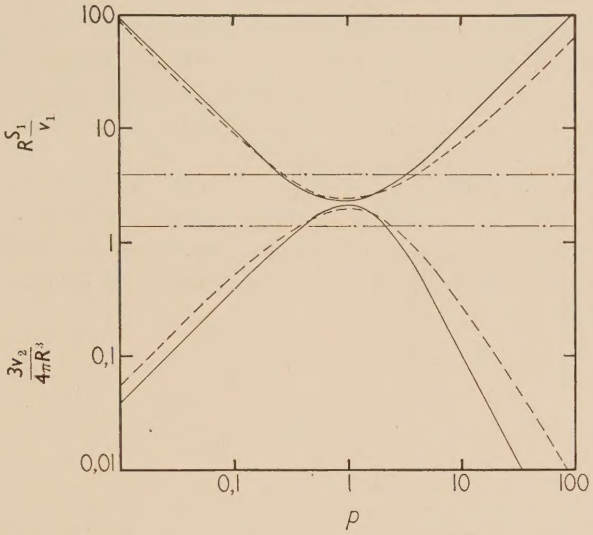

FIG. 1. Abaques relatifs aux ellipsoïdes de révolution. En trait plein $3v_1/4\pi R_0^3$ et $R_0 S_1/v_1$; en trait interrompu $3v_1/4\pi R_a^3$ et $R_a S_1/v_1$.

dernière: la surface, en effet, est bien plus sensible que le rayon de giration et le volume à la présence de protubérances et de plissements. En conséquence on peut prévoir que l'ellipsoïde correspondant au paramètre $R_0 \frac{S_1}{v_1}$ soit en général plus anisodiamétrique que celui correspondant à $3v_1/4\pi R_0^3$.

3. Étude du Lysozyme

(a) *Revue bibliographique*

La composition en acides aminés du lysozyme a été étudiée surtout par Fromageot et son école (Jolles, Jolles-Thaureaux & Fromageot, 1958): la masse moléculaire, déterminée par voie chimique, est 14.700 $\pm$ 250 (Fromageot & Privat de Garilhe, 1950).†

La masse moléculaire a été déterminée également par diffusion de la lumière: Halver, Nutting & Brice (1951) ont obtenu $M = 14.800$.

L'étude de la structure cristalline a été entreprise il y a déjà plusieurs années (Corey, Donohue & Trueblood, 1952); Palmer, Ballantyne & Galvin (1948) ont déterminé la masse moléculaire ($M = 13.900 \pm 600$) dans les cristaux de chlorure de lysozyme.

Ritland, Kaesberg & Beeman (1950) ont étudié le lysozyme en solution par diffusion centrale des rayons X; ils ont obtenu $R_0 = 16{,}0$ Å, et ils ont montré que la forme des

† Note ajoutée en cours d'impression : Jollés, Jollés & Jaurequi-Adell (1960), ont précisé récemment les analyses de Fromageot & Privat de Garilhe (1950); la masse trouvée est de 14.095.

courbes expérimentales est compatible avec deux ellipsoïdes de révolution, l'un aplati $p = 0{,}30$, l'autre allongé $p = 2{,}7$.

Les données physiques sont peu nombreuses, et assez discordantes entre elles (Edsall, 1953); l'étude la plus complète est celle de Luzzati & Champagne (1957). Nous analyserons plus loin les résultats de ce dernier travail.

(b) *Résultats expérimentaux*

La protéine utilisée dans ce travail provient du blanc d'oeuf de poule: elle nous a été fournie par la Cie Armour (lot no. D. 638.040). Nous en avons vérifié la pureté par sédimentation ($S = 1{,}90.10^{-13}$). Toutes les expériences ont été effectuées en solution dans l'eau salée (0,15 M-ClNa), à un pH voisin de 5,7 et à température ambiante (environ 25°C): la densité électronique du solvant est 0,335 él. Å^{-3}. La concentration des solutions a été fixée par pesée, après qu'on ait établi la teneur en eau et en azote de la protéine séchée à l'air, et a été vérifiée avant et après l'expérience de rayons X par des mesures d'absorption dans l'ultraviolet: nous avons admis que la teneur en azote de la protéine séche est 18,6%, et que le coefficient d'absorption à 280 mμ est 26,4/cm/1%
(Fromageot & Schneck, 1950).

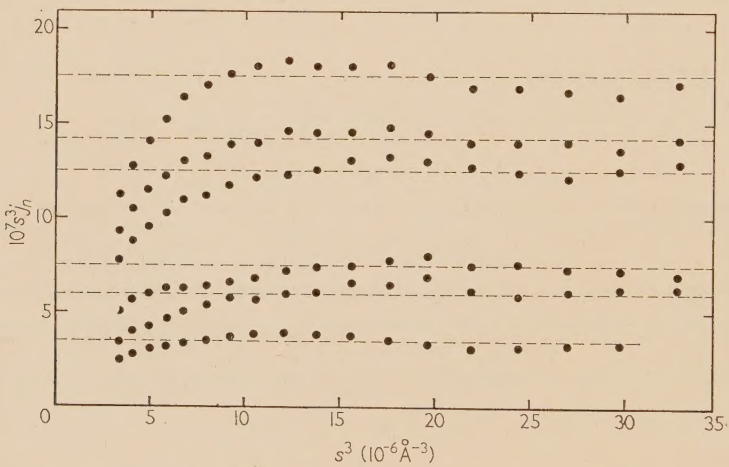

FIG. 2. Représentation des points expérimentaux $s^3 j_n(s)$, en fonction de s^3.

Le volume spécifique partiel a été mesuré par Charlwood (1957): $\bar{v} = 0{,}7138$ cm^3. g^{-1}. Le rapport entre masse molaire et nombre d'électrons est 1,88 (Fromageot & Privat de Garilhe, 1950). Avec ces valeurs numériques le volume spécifique électronique et le rapport entre la concentration pondérale et la concentration électronique sont:

$$\psi = 2{,}227 \text{ Å}^3 \text{ él.}^{-1}, \; c_e = 0{,}957 \; c \; (1 - 0{,}45c)^{-1}.$$

Nous avons effectué plusieurs séries d'expériences de diffusion centrale avec cette protéine, en utilisant, une technique photographique (Champagne, Luzzati & Nicolaieff, 1961), et en nous servant, plus récemment, de mesures absolues, selon la technique décrite ci-dessus: les résultats de toutes ces expériences sont semblables. Nous décrirons ici la série la plus complète de mesures effectuées à l'échelle absolue, qui porte sur six solutions, à des concentrations allant de 1% à 5%.

Nous avons vérifié si les données expérimentales sont compatibles avec le modèle d'un milieu à deux densités (voir Appendice).

Les points $s^3 j_n(s)$ sont représentés dans Fig. 2, en fonction de s^3: pour s grand les points s'alignent sur des droites horizontales. L'expérience satisfait donc aux exigeances du modèle. Les valeurs de $\lim_{s \to \infty} s^3 j_n(s)$ sont mesurées par l'ordonnée à l'origine des droites de Fig. 2; les valeurs de δ^* sont ici négligeables.

La représentation de Guinier des points expérimentaux relatifs aux petites valeurs de s donne des droites (Fig. 3), qui permettent d'extrapoler $j_n(s)$ vers l'origine, et de déterminer R_a (2).

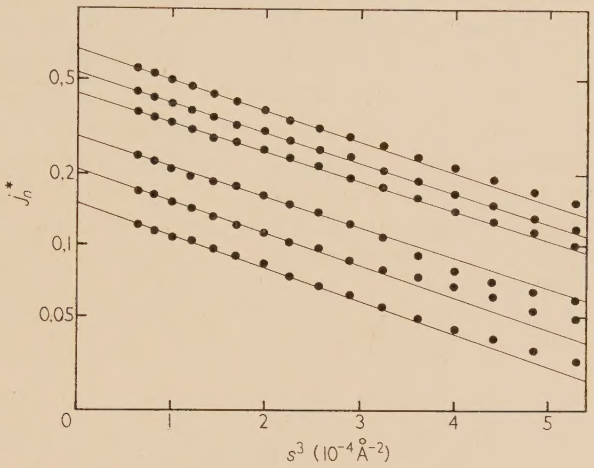

Fig. 3. Représentation de Guinier.

Ayant ainsi extrapolé les courbes $j_n(s)$ vers $s = 0$ et vers $s = \infty$, on peut déterminer $\phi(s)$ (2), et on peut calculer tous les termes des équations (3), (4), (5), (10), (11), 12); on peut déterminer ensuite la valeur des différents paramètres R_a, R_0, $i_n(0)$, $\rho_1 - \rho_0$, $\frac{S_1}{v_1}$. Les contributions de $\phi(s)$ à R_a et $i_n(0)$ sont inférieures à 1% de toutes les concentrations: nous nous les avons négligées.

(c) *Discussion*

Les valeurs numériques des différents paramètres expérimentaux sont portées dans le Tableau 1 et dans Fig. 4.

Tableau 1

Résultats expérimentaux

c%	1,01	1,45	2,07	3,01	3,84	4,95
$j_n(0)$	0,149	0,209	0,288	0,440	0,537	0,670
R_0 (Å)	15,2	15,4	15,2	14,9	15,1	15,3
$10^7 \lim_{s \to \infty} [s^3 j_n(s)]$	3,50	6,00	7,50	12,50	14,25	17,50
$10^6 \int_0^\infty 2\pi s j_n(s) ds$	219	340	440	711	842	1,042
$i_n(0)$	4,63	6,58	8,96	13,41	16,59	20,97
M	13.940	13.810	13.170	13.550	13.150	12.890
$(\rho_1 - \rho_0)$(él. Å^{-3})	9,00	9,75	9,00	9,96	9,33	9,05
$\frac{S_1}{v_1}$(Å^{-1})	0,250	0,275	0,262	0,271	0,258	0,253

M et R_0 varient avec la concentration: nous les avons extrapolés linéairement à $c = 0$ par un calcul de moindres carrés. Au contraire, et en accord avec la théorie, S_1/v_1 et $(\rho_1 - \rho_0)$ sont indépendants de la concentration, du moins dans la limite des erreurs expérimentales: les valeurs adoptées sont des moyennes pondérées par la concentration.

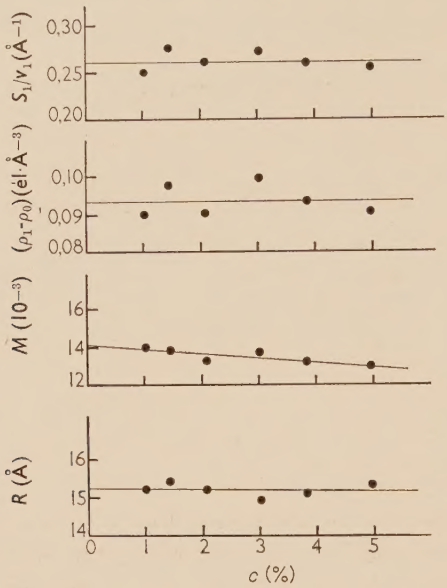

Fig. 4. Valeurs des différents paramètres, en fonction de la concentration, et droites obtenues par le calcul des moindres carrés.

Les valeurs des paramètres relatifs à la particule (donc extrapolés, le cas échéant, à $c = 0$) sont portées dans le Tableau 2, où figurent également, entre parenthèses, les erreurs quadratiques moyennes obtenues par le calcul des moindres carrés.

Tableau 2

Paramètres de la particule

$R_0 = R_a$ (Å)	15,2	($\pm$ 0,2)
M	14.100	($\pm$ 200)
$(\rho_1 - \rho_0)$ (él. Å^{-3})	0,093	($\pm$ 0,003)
$\frac{S_1}{v_1}$ (Å^{-1})	0,260	($\pm$ 0,007)
v_1 (Å^3)	20.400	
α	0,17	$\left(0{,}18 \frac{\text{g } H_2O}{\text{g protéine}}\right)$

La masse, déterminée par diffusion centrale des rayons X, est légèrement plus petite que la masse déterminée par voie chimique: la différence, toutefois, dépasse à peine les limites d'incertitude inhérentes aux erreurs des deux mesures

$$\frac{M_{\text{chimique}}}{M_{\text{R.X.}}} = \frac{14.700 \pm 250}{14.100 \pm 200} = 1{,}042 \pm 0{,}023$$

L'écart pourrait être dû à des erreures, ou bien à une perturbation de la distribution du ClNa dans le solvant, au voisinage des molécules de protéine (Luzzati *et al.*, 1961).

La valeur de rayon de giration est nettement plus petite que celle déterminée par Ritland *et al.* (1950): 15,3 au lieu de 16,0 Å. La mesure de R_0 est très précise, donc il semble que cet écart tienne à une différence entre les deux échantillons de lysozyme.

Tableau 3

Modèles d'ellipsoïdes

$\frac{3v_1}{4\pi R_0^3}$	$R_0\frac{S_1}{v_1}$	p	a(Å)	b(Å)	D (10^{-7})	$[\eta]$
1,39		0,38	8,8	23,3	12,0	2,79
		2,2	28,7	13,0	11,9	2,62
	3,95	0,29	6,8	23,6	11,1	3,26
		3,3	31,3	9,5	11,1	3,40
			Valeurs expérimentales		11,8	2,98

Ayant déterminé cet ensemble de paramètres, nous avons cherché à les interpréter en admettant que la particule ait la forme d'un éllipsoïde de révolution. Nous avons calculé (Tableau 3) les valeurs de $3v_1/4\pi R_0^3$ et de R_0S_1/v_1, et nous les avons portées sur les abaques (Fig. 1): chaque abaque fournit deux valeurs de p, une correspondant à un ellipsoïde aplati, l'autre à un ellipsoïde allongé. L'accord entre les valeurs de p relatives aux deux abaques est satisfaisant: le sens de l'écart suggère, en outre, que la surface de la particule n'est pas aussi lisse que l'enveloppe ellipsoïdale (voir ci-dessus). Il est impossible toutefois, de choisir entre l'ellipsoïde aplati et l'ellipsoïde allongé. Les valeurs de p sont en outre en bon accord avec celles proposées par Ritland *et al.* (1950).

Nous avons vérifié également si les dimensions des ellipsoïdes qu'on obtient par diffusion centrale des rayons X sont compatibles avec les propriétés hydrodynamiques du lysozyme en solution. Pour cela nous avons calculé les valeurs de la viscosité intrinsèque et de la constante de diffusion de translation, en utilisant les formules théoriques, relatives aux ellipsoïdes de révolution (Sadron, 1953):

$$[\eta] = \frac{Nv_1}{M} A(p) \tag{18}$$

$$D = \frac{1{,}612kT}{6\pi\eta_0 v_1^{\frac{1}{3}}} t(p) \tag{19}$$

où M est la masse molaire de la protéine sèche (14.100), v_1 est le volume de la particule gonflée de solvant (20.400 Å^3), N est le nombre d'Avogadro, η_0 est la viscosité du solvant, et $A(p)$ et $t(p)$ sont des fonctions connues. Les résultats des calculs sont portés dans le Tableau 3, où figurent également les valeurs expérimentales obtenues par Luzzati & Champagne (1957). L'accord est excellent, aussi bien pour un ellipsoïde allongé ($p = 2{,}5$) que pour un ellipsoïde aplati ($p = 0{,}35$).

En ce qui concerne la diffusion centrale des rayons X cet accord indique d'une part que le modèle d'un milieu à deux densités convient dans le cas des protéines, et justifie,

d'autre part, la référence à un ellipsoïde de révolution pour décrire la forme extérieure de la particule.

En outre, et cela est peut-être plus important, ces résultats suggèrent que les lois de l'hydrodynamique des milieux continus sont encore valables à l'échelle moléculaire.

Appendice

(a) *La forme de $j_n(s)$, pour s grand*

L'existence des comportements asymptotiques de $i_n(s)$ et de $j_n(s)$ (voir L),

$$\lim_{s\to\infty} \overline{s^4 i_n(s)} = \frac{1}{8\pi^3}\frac{S_1}{v_1}(\rho_1 - \rho_0)c_e(1 - \rho_0\psi) \tag{20}$$

$$\lim_{s\to\infty} s^3 j_n(s) = \frac{1}{16\pi^2}\frac{S_1}{v_1}(\rho_1 - \rho_0)c_e(1 - \rho_0\psi) \tag{21}$$

n'a été prouvée, en toute rigueur, que dans le cas où la densité électronique au sein de l'échantillon ne prend que deux valeurs, ρ_1 et ρ_0: or il est clair qu'un tel modèle ne peut décrire la structure des solutions de protéines que de manière approchée, car la densité électronique n'est parfaitement constante ni dans le solvant, ni, à plus forte raison, à l'intérieur des molécules de protéine. Un modèle plus satisfaisant est celui où la densité électronique présente, à courte distance, des fluctuations autour d'une valeur moyenne qui, elle, est uniforme aussi bien dans le soluté que dans le solvant. On peut, dans ce cas, analyser l'influence des fluctuations sur la forme asymptotique de $i_n(s)$ et de $j_n(s)$.

On calcule $i_n(s)$ en dehors de la région des petits angles en soustrayant de l'intensité diffusée par la solution l'intensité que diffuse le même volume de solvant;

$$i_n(s) = \frac{i_1(s) - i_0(s)c_e\rho_0\psi}{V\bar{\rho}} \tag{22}$$

Les indices 1 et 0 se réfèrent respectivement à la protéine et au solvant: $c_e\psi\rho_0$ est la fraction d'électrons du solvant remplacée par les électrons du soluté.

Si on appelle $\epsilon_1(s)$ le rapport entre l'intensité diffusée par la protéine et l'intensité que diffuseraient les mêmes atomes à l'état gazeux, $\epsilon_0(s)$ le même rapport relatif au solvant, et si $f_j(s)$ et Z_j sont le facteur de structure et le numéro atomique de l'atome j, N le nombre de molécules de protéine de l'échantillon, on a (Luzzati, 1955):

$$\frac{i_1(s)}{V\bar{\rho}} = \frac{N\epsilon_1(s)\sum_{j=1}^{n} f_j^2(s)}{\frac{1}{c_e}N\sum_{j=1}^{n} Z_j} = c_e\epsilon_1(s)\left(\frac{\overline{f^2(s)}}{\overline{Z}}\right)_1 \tag{23}$$

$$\frac{i_0(s)}{V\bar{\rho}} = (1 - c_e)\epsilon_0(s)\left(\frac{\overline{f^2(s)}}{\overline{Z}}\right)_0 \tag{24}$$

$$i_n(s) = c_e\left[\epsilon_1(s)\left(\frac{\overline{f^2(s)}}{\overline{Z}}\right)_1 - (1 - c_e)\rho_0\psi\epsilon_0(s)\left(\frac{\overline{f^2(s)}}{\overline{Z}}\right)_0\right] \tag{25}$$

Dans la région immédiatement voisine du domaine des "petits angles", c'est-à-dire, dans les conditions de nos expériences, aux environs de $s = 0{,}1$ Å^{-1}, on peut admettre, du moins en première approximation, que $\epsilon(s)$ et $f(s)$ sont indépendants de s. Par ailleurs

la hauteur de collimation cesse d'être "infinie" dans cette région. On peut alors calculer $j_n(s)$ par l'expression approchée:

$$\begin{aligned} j_n(s) &= \int_{-t_1}^{+t_1} i_n(\sqrt{s^2+t^2})dt \simeq 2t_1 i_n(s) \\ &= 2t_1 c_e \left[\epsilon_1(s) \left(\frac{\bar{Z}^2}{\bar{Z}} \right)_1 - (1 - c_e)\rho_0 \psi \epsilon_0(s) \left(\frac{\bar{Z}^2}{\bar{Z}} \right)_0 \right] \\ &= \delta^* \end{aligned} \tag{26}$$

Les fonctions $\epsilon(s)$ ont été déterminées par Arndt & Riley (1955) pour plusieurs protéines, et par Morgan & Warren (1938) pour l'eau: pour $s \simeq 0{,}1$ Å^{-1} on trouve 0,5, $< \epsilon_1 < 0{,}9$ et $\epsilon_0 \simeq 0{,}2$. La valeur de ψ est dans les protéines voisine de 2,3 Å^3 él.$^{-1}$; $\bar{Z}^2/\bar{Z}$ est voisin de 7 pour les protéines, de 10 pour l'eau. La valeur de t_1 dépend des conditions expérimentales: dans nos montages t_1 est compris entre 0,07 Å^{-1} et 0,12 Å^{-1}. Avec ces valeurs numériques, et à des concentrations suffisemment faibles pour que c_e soit négligeable devant 1, δ^* est compris entre $0{,}30c_e$ et $0{,}10c_e$.

En première approximation les fluctuations à courte distance ajoutent donc une constante δ^* au terme en s^{-3} de l'expression asymptotique de $j_n(s)$

$$\lim_{s \to \infty} s^3 j_n(s) = \frac{1}{16\pi^2} \frac{S_1}{v_1} (\rho_1 - \rho_0) c_e (1 - \rho_0 \psi) + \delta^* s^3 \tag{27}$$

Ce terme δ^* est toujours très petit devant $j_n(0)$ (voir les résultats expérimentaux relatifs à la sérumalbumine de boeuf).

En fait la courbe expérimentale $j_n(s)$ n'est pas connue avec grande précision, pour s grand, et les erreurs expérimentales peuvent parfois être de grandeur comparable à δ^*: formellement δ^* peut donc rendre compte à la fois des fluctuations de la densité électronique et de certaines erreurs systématiques de $j_n(s)$. Une difficulté peut provenir des oscillations de $j_n(s)$, autour de la courbe théorique (2), dues à la forme des particules; le calcul montre, du moins dans le cas des ellipsoïdes de révolution (Malmon, 1957) que ces oscillations sont d'autant plus prononcées que la forme est voisine d'une sphère. Lorsque ces oscillations sont grandes il peut devenir malaisé sinon impossible, de reconnaître dans la courbe $j_n(s)$ la partie en s^{-3}.

Du point de vue pratique on peut analyser le comportement asymptotique de $j_n(s)$ en traçant la courbe expérimentale $s^3 j_n(s)$ en fonction de s^3: si les points s'alignent, pour s grand, sur une droite, on obtient (27)

$$\delta^* \quad \text{et} \quad \frac{1}{16\pi^2} \frac{S_1}{v_1} (\rho_1 - \rho_0) c_e (1 - \rho_0 \psi)$$

Lorsque δ^* est connu il convient de le soustraire de $j_n(s)$ pour toutes les valeurs de s:

$$j_n^*(s) = j_n(s) - \delta^* \tag{28}$$

et de poursuivre les calculs avec cette fonction $j_n^*(s)$, au lieu de $j_n(s)$, car la valeur de δ^* est, en première approximation, indépendante de s. Dans la pratique δ^* s'est toujours avéré très petit devant $j_n(0)$, et l'influence de la soustraction de δ^* est sensible seulement sur les valeurs des paramètres liés au modèle d'un milieu à deux densités (éq. 10, 11, 12): les valeurs de la masse et du rayon de giration sont les mêmes, qu'on les détermine avec $j_n(s)$ ou avec $j_n^*(s)$.

Nous tenons à exprimer notre reconnaissance à Monsieur H. Benoit et Monsieur M. Daune, et à Mademoiselle M. Champagne, qui ont suivi notre travail avec intérêt, en nous aidant souvent de leurs conseils.

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La Structure de la Sérum Albumine de Boeuf en Solution à pH 5,3 et 3,6 : Étude par Diffusion Centrale Absolue des Rayons X

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The reversible denaturation of bovine serum albumin (BSA) at acidic pH has been studied by small-angle X-ray scattering experiments, performed on an absolute scale.

Near the isoionic point (pH 5·3) BSA is a compact molecule; the molecular weight, radius of gyration, volume, external surface and internal hydration have been determined. All these parameters agree closely with results obtained with other techniques.

Important changes in X-ray diffraction are observed at pH 3·6.

1. The intensity varies evenly over a large range of diffraction angle, as it does at pH 5·3, but at very small angles it suddenly rises to large values.

2. The radius of gyration increases from 30·6 Å to 68·5 Å.

3. The molecular weight does not change.

4. The molecule swells, but the experimental observations are not compatible with a uniform compact particle.

Several possible interpretations of these observations are discussed and a model which explains satisfactorily the mechanism of the transformation is proposed. The molecule is a compact particle at isoionic pH. At acidic pH one part of the polypeptide chain (about 35%) unravels, and assumes a loose random-coil like structure; the rest of the molecule remains folded in a compact particle.

This model is shown to agree quantitatively with the X-ray experiments, and the dimensions of both the compact nucleus and the loose chain are determined.

Other experimental observations (namely hydrodynamic properties and optical rotation measurements) are discussed, and are shown to be consistent with the model.

1. Introduction

Le nombre des mémoires consacrés aux propriétés de la sérumalbumine de boeuf (SAB) en solution aqueuse est impressionnant: c'est en particulier une propriété singulière de cette protéine, sa "dénaturation réversible", qui a suscité la plupart de ces travaux, du moins au cours des dernières années. Ce phénomène, qui consiste en une modification structurale profonde, et complètement réversible, induite par certains changements du solvant (pH, force ionique, urée), a été étudié par de nombreuses techniques; plusieurs interprétations en ont été proposées sans que l'accord entre les différents auteurs soit encore unanime (Foster, 1960).

Au Centre de Recherches sur les Macromolécules, Champagne s'occupe de ce problème depuis plusieurs années (Champagne & Sadron, 1955; Champagne, 1957) en étudiant

surtout le comportement hydrodynamique de la SAB en fonction du pH. C'est en collaboration avec Champagne que nous avons effectué, il y a quelques années, l'étude morphologique de la SAB par diffusion centrale des rayons X, en utilisant une technique photographique; nous avons publié certains résultats (Nicolaieff, 1958; Champagne, Luzzati & Nicolaieff, 1958) et nous avons proposé un mécanisme de dénaturation réversible, qui rend compte de manière satisfaisante aussi bien des expériences de diffusion des rayons X que des propriétés hydrodynamiques.

La technique de la diffusion centrale absolue, que nous avons mise au point récemment (Luzzati, 1960; Luzzati, Witz & Nicolaieff, 1961, abrégés par L et LWN dans la suite), se prête tout particulièrement pour étudier ce phénomène, et pour en préciser le mécanisme: c'est dans ce but que nous avons entrepris le travail décrit ici. Les conditions expérimentales nous ont été suggérées par le travail de Champagne (1957): nous nous sommes placés à pH 5,3 et à pH 3,6, valeurs qui se trouvent à l'intérieur du domaine de réversibilité, et pour lesquelles la protéine adopte deux configurations différentes et caractéristiques. Nous avons effectué plusieurs séries d'expériences; nous en décrirons deux, choisies parmi les plus complètes.

En fait, malgré le volume de travail qui lui a été consacré, la SAB est à bien des égards un choix peu heureux: on ne connaît pas, en effet, de propriété biochimique spécifique qui permette de reconnaître l'état "natif" et l'état "dénaturé" de cette protéine, les propriétés physicochimiques varient souvent d'un lot à l'autre (Champagne, 1957) et les échantillons ne sont pas rigoureusement monodisperses (Champagne, Jung & Daune, 1957). Dans ces conditions les résultats qu'on obtient ne peuvent pas être aussi sûrs et précis que ceux que la même technique peut fournir avec des substances mieux caractérisées.

2. Partie Experimentale

La SAB nous a été fournie par la Cie Armour. Toutes les expériences décrites dans ce mémoire ont été effectuées avec un même lot (lot no. 67808, liste 2267).

Nous avons déterminé la masse de la protéine, par diffusion de la lumière, à pH 5,3, dans l'eau salée (NaCl, 0,15 M): $M = 75.200$. La constante de sédimentation, dans l'eau salée, à 20°C, à une concentration $c = 0{,}75\%$ (pH = 5,3) est $S = 4{,}1.10^{-13}$. En outre nous avons décelé un composant lourd dont nous avons estimé la concentration à 10% environ (Champagne, 1957; Champagne *et al.*, 1957). Nous avons effectué également quelques contrôles, par sédimentation, des solutions avant et après les expériences de rayons X: nous n'avons mis en évidence aucune différence.

La solution mère de protéine servant aux expériences à pH = 3,6 a été délipidée en la conservant à pH $\simeq$ 3 durant trois jours, à 4°C; le précipité a été éliminé par centrifugation (ou filtration), et le pH a été ensuite ramené à pH = 3,6 (Sterman & Foster, 1956).

Les concentrations ont été déterminées par des mesures d'absorption dans l'ultraviolet après les expériences de rayons X: $E = 6{,}67/\%/\text{cm}$ à 279 mμ (Sterman & Foster, 1956).

Le pH a été modifié en ajoutant aux solutions de SAB une solution de HCl (0,15 M): le pH a été contrôlé au début et à la fin des expériences. Toutes les solutions contiennent du ClNa, 0,15 M.

Le rapport entre la masse molaire et le nombre d'électrons est 1,87 (Edsall, 1953): le volume spécifique est $\bar{v} = 0{,}734\ \text{g}^{-1}\,\text{cm}^3$ aussi bien à pH 5,3 qu'à pH 3,6 (Charlwood, 1957). Le volume spécifique électronique et la concentration électronique sont:

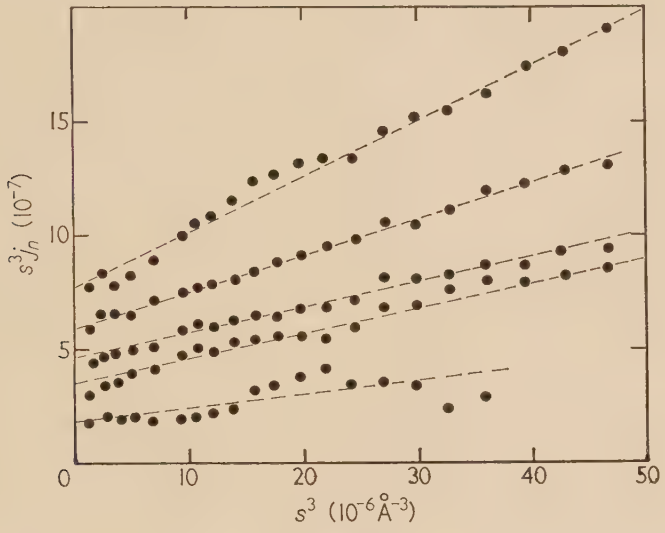
$$\psi = 2{,}283\ \text{él.}^{-1}\,\text{Å}^3\ ; \quad c_e = 0{,}960\ c[1 - 0{,}042\ c]^{-1}$$

Toutes les expériences ont été effectuées à une temperature voisine de 25°C.

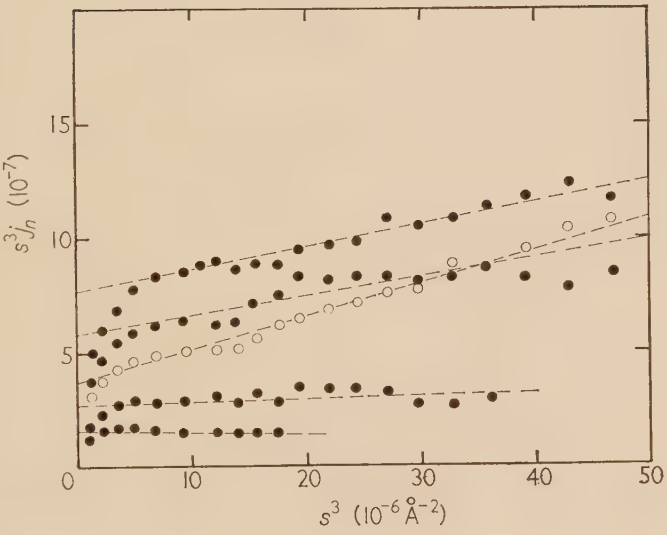
La technique expérimentale a été décrite ailleurs (L, LWN).

(a) *Point isoionique: pH 5,3*

Nous avons représenté dans Fig. 1(a) les points expérimentaux $s^3 j_n(s)$, en fonction de s^3: les points s'alignent de manière satisfaisante sur des droites, ce qui est en bon accord



(a) pH 5,3



(b) pH 3,6

Fig. 1. Points expérimentaux $s^3 j_n(s)$, en fonction de s^3.

avec le modèle d'un milieu à deux densités. Nous avons ainsi déterminé les deux termes du développement asymptotique de $j_n(s)$ (LWN, éq. 27), que nous avons portés dans le Tableau 1. δ^* est proportionnel à la concentration ($\delta^* \simeq 0{,}42\, c_e$), du moins en première

approximation, et la constante de proportionnalité est en bon accord avec la valeur théorique. Nous avons soustrait δ^* de $j_n(s)$:

$$j_n^*(s) = j_n(s) - \delta^*$$

ce sont ces fonctions $j_n^*(s)$ que nous utilisons dans la suite (voir appendice dans LWN).

Tableau 1

Résultats expérimentaux à pH 5,3

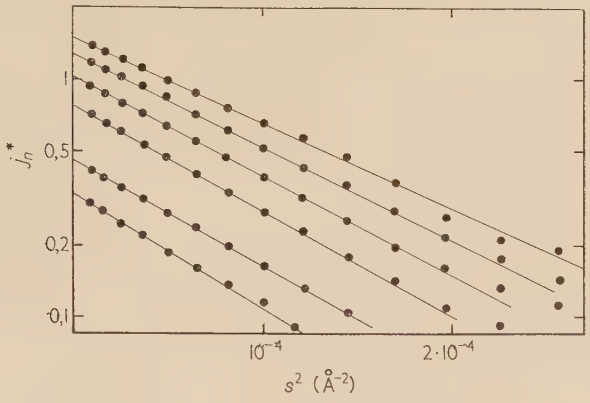
$c\%$	0,93	1,34	2,10	3,07	4,08	5,21
δ^*	0	0,006	0,011	0,011	0,016	0,025
$j_n^*(0)$	0,333	0,457	0,778	1,010	1,285	1,480
R_a (Å)	29,80	28,25	28,35	27,19	26,57	24,97
$10^7 \lim [s^3 j_n^*(s)]$	—†	1,9	3,5	4,6	5,9	7,8
$10^6 \cdot 2\pi \int_0^\infty s_1 j_n^*(s) ds$	—†	198	358	483	631	793
$\int_0^\infty s^{-2} \phi(s) ds$	—†	0,460	1,393	1,298	1,667	1,233
$\int_0^\infty s^{-4} \phi(s) ds$	—†	13.000	6.340	6.430	8.830	6.570
R_0 (Å)	30,0†	28,6	28,6	27,4	26,8	25,1
i_n (0)	20,10†	26,30	44,69	55,78	69,32	75,22
M	76.465	69.050	74.910	64.040	59.735	50.830
$(\rho_1 - \rho_0)(10^{-2}$. él. Å$^{-3})$	—	6,64	7,69	7,20	7,14	7,13
$\frac{S_1}{v_1}$ (Å^{-1})	—	0,150	0,151	0,146	0,141	0,147

† L'intensité diffusée par cette solution était trop faible dans la région des angles relativement grands ($2\theta > 1°$). La correction $\phi(s)$ a été déterminée d'après la moyenne des corrections des autres concentrations.

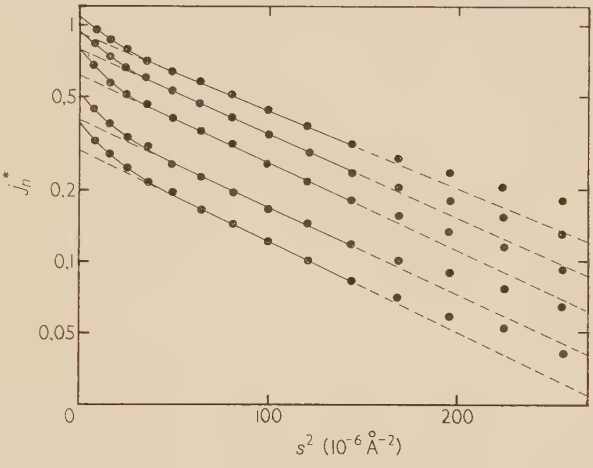
Les courbes $j_n^*(s)$ sont portées dans Fig. 2, selon la représentation de Guinier: on obtient ainsi des droites qui permettent d'extrapoler $j_n^*(s)$ vers l'origine et de déterminer R_a (LWN, éq. 2). En soustrayant la fonction $j_n^*(0) \exp\left(-\frac{4\pi^2}{3} R_a^2 s^2\right)$ de chaque courbe $j_n^*(s)$ nous avons obtenu les fonctions $\phi(s)$, et nous avons pu calculer tous les termes des équations (LWN, 3, 4, 5, 11, 12). Nous avons déterminé ensuite les valeurs des différents paramètres relatives à chaque concentration (Tableau 1, et Fig. 3).

$i_n(0)$, R_a et R_0 varient avec la concentration: nous les avons extrapolés linéairement vers $c = 0$ par un calcul des moindres carrés. $(\rho_1 - \rho_0)$ et S_1/v_1, au contraire, ne dépendent pas de la concentration, en bon accord avec la théorie. Les valeurs de la masse M, des rayons de giration R_a et R_0, du contraste de densité électronique $(\rho_1 - \rho_0)$, de la surface spécifique S_1/v_1, du volume v_1 et du taux d'hydratation interne α sont portées dans le Tableau 3: entre parenthèses figure l'erreur quadratique moyenne, obtenue par le calcul des moindres carrés.

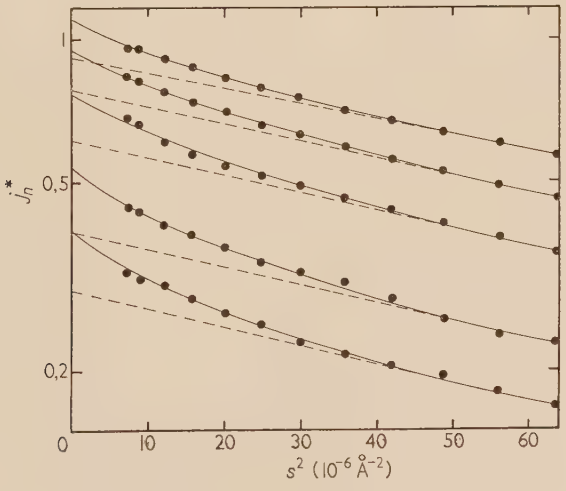
Nous avons calculé également les paramètres $3v_1/4\pi R_0^3$ et $R_0 S_1/v_1$, et nous avons cherché à déterminer, au moyen des abaques, les dimensions de l'ellipsoïde qui rend le mieux compte des résultats expérimentaux (Fig. 4 et Tableau 4). L'accord entre les deux abaques est satisfaisant: comme dans le cas du lysozyme, l'écart est dans le sens prévu, si on suppose la présence d'accidents à la surface de la particule. Il est difficile de choisir entre un ellipsoïde aplati ($p \simeq 1/3$) et un ellipsoïde allongé ($p \simeq 2{,}5$).



(a) pH 5,3



(b) pH 3,6



(c) pH 3,6, échelle de s dilatée

en trait plein: $A \exp\left(-\frac{4\pi^2}{3}\alpha^2 s^2\right) + B \exp\left(-\frac{4\pi^2}{3}\beta^2 s^2\right)$

en pointillé: $A \exp\left(-\frac{4\pi^2}{3}\alpha^2 s^2\right)$

FIG. 2. Représentation de Guinier.

(b) *pH 3,6*

Nous avons interprété les courbes expérimentales par le procédé décrit précédemment. Les points $s^3 j_n(s)$, représentés en fonction de s^3, s'alignent, pour s grand, sur des droites (Fig. 1(b)), dont la pente varie régulièrement avec la concentration (avec l'exception, toutefois, de la courbe relative à $c = 3{,}31\%$, qui est affectée vraisemblablement d'une petite erreur, indépendante de s).

Nous avons tracé dans Fig. 2(b) les courbes expérimentales dans la représentation de Guinier. Les courbes ont une forme différente de celle qu'on rencontre habituellement dans le cas des particules globulaires: en venant des grands angles vers l'origine, les points expérimentaux tendent d'abord régulièrement vers une droite, avec laquelle ils se confondent entre $s \simeq 11.10^{-3}$ Å^{-1} et $s \simeq 7.10^{-3}$ Å^{-1}, puis, au voisinage de l'origine, ils s'en écartent brusquement. Nous reviendrons plus loin sur l'interprétation de cette anomalie. L'extrapolation de $j_n^*(s)$ vers l'origine devient, dans ce cas, délicate: nous nous sommes aidés d'un artifice mathématique. Nous avons constaté, en effet, que la différence entre les courbes $j_n^*(s)$ et la fonction de Gauss avec lesquelles elles se confondent entre $s \simeq 11.10^{-3}$ Å^{-1} et $s \simeq 7.10^{-3}$ Å^{-1}, est elle même une fonction de Gauss (Fig. 2(c)): nous avons alors décomposé $j^*(s)$ en trois termes

$$j_n^*(s) = A \exp\left(-\frac{4\pi^2}{3}\alpha^2 s^2\right) + B \exp\left(-\frac{4\pi^2}{3}\beta^2 s^2\right) + \phi(s) \tag{1}$$

et nous avons admis que l'équation (1) décrit la forme de $j_n^*(s)$ jusqu'à $s = 0$. Dans ces conditions les expressions de $i_n(0)$ et de R_0 sont (Luzzati, 1958):

$$i_n(0) = 2\sqrt{\frac{\pi}{3}}(A\alpha + B\beta) - \frac{1}{\pi}\int_0^\infty s^{-2}\phi(s)\mathrm{d}s \tag{2}$$

$$R_0^2 = \frac{3}{4\pi^2}\left[\frac{8\pi^2}{3}\sqrt{\frac{\pi}{3}}(A\alpha^3 + B\beta^3) + \frac{3}{2\pi}\int_0^\infty s^{-4}\phi(s)\mathrm{d}s\right]\left[i_n(0)\right]^{-1} \tag{3}$$

Les valeurs des différents termes relatifs aux équations 1, 2, 3, et les valeurs des paramètres R_0, M, $(\rho_1 - \rho_0)$ et S_1/v_1 sont portées dans le Tableau 2 et dans Fig. 3. Les extrapolations linéaires à $c = 0$ ont été faites par un calcul des moindres carrés.

On constate tout d'abord que la masse est la même à pH 3,6 et 5,3, en bon accord avec les mesures de diffusion de la lumière; ce fait justifie en outre l'extrapolation de $j_n^*(s)$ vers l'origine par l'équation (1).

La masse, toutefois, est le seul paramètre qui reste constant aux deux valeurs du pH: les changements des autres paramètres sont importants. Il faut noter, en outre, que $(\rho_1 - \rho_0)$ (Fig. 3 et Tableau 2) varie régulièrement avec la concentration, ce qui est en désaccord à la fois avec les exigeances du modèle d'un milieu à deux densités, et avec les observations expérimentales relatives au lysozyme et à la SAB au point isoionique.

En négligeant cette anomalie, et en utilisant les valeurs des paramètres extrapolées à $c = 0$, nous avons cherché à déterminer la forme et les dimensions de l'ellipsoïde de révolution compatible avec nos observations expérimentales. Cet ellipsoïde est très anisodiamétrique, qu'il soit aplati ou allongé (Fig. 4 et Tableau 4): la forme de la molécule devient ici plus voisine d'un bâtonnet ou d'une plaquette que d'une particule globulaire. Qu'il s'agisse d'ailleurs d'un ellipsoïde, ou de toute autre forme compacte, le rapport entre le volume et le rayon de giration suffit à indiquer que la particule doit être fortement anisodiamétrique.

Tableau 2

Résultats expérimentaux à pH = 3,6

$c\%$	1,59	2,27	3,31	4,16	5,34
δ^*	− 0,001	+ 0,001	+ 0,014	+ 0,008	+ 0,009
A	0,295	0,395	0,616	0,782	0,920
α (Å)	26,08	25,86	25,61	25,03	24,06
B	0,102	0,144	0,155	0,168	0,180
β (Å)	81,95	77,67	73,16	72,05	69,98
$10^7 \lim [s^3 j_n^*(s)]$	1,6	2,7	3,7	5,8	7,7
$10^6 \int_0^\infty 2\pi s j_n^*(s) ds$	163	245	358	499	641
$\int_0^\infty s^{-2}\phi(s) ds$	0,614	1,152	1,369	1,964	2,216
$\int_0^\infty s^{-4}\phi(s) ds$	3.725	7.180	8.260	10.680	12.150
R_0 (Å)	62,06	59,97	51,51	49,06	46,70
i_n (0)	32,65	42,44	55,04	64,19	70,35
M	72.250	65.620	58.410	54.340	46.350
$(\rho_1 - \rho_0)(10^{-2}$ él. Å$^{-3})$	4,65	4,95	5,02	5,61	5,74
$\frac{S_1}{v_1}$ (Å^{-1})	0,151	0,168	0,155	0,173	0,176
$[R_0]_c$ (Å)	26,2	26,3	26,0	25,4	24,4
$[i_n\ (0)]_c$	15,55	20,53	31,84	39,42	44,58
M_c	49.860	45.640	44.430	42.580	36.890
M_1	22.390	19.980	13.980	11.760	9.460
$k = M_c/M$	0,690	0,696	0,761	0,784	0,796
$[R_0]_1$ (Å)	104,4	101,2	94,6	93,7	91,4
$10^6 \int_0^\infty 2\pi s [j_n^*(s)]_c ds$	159	240	351	491	632
$[\rho_1 - \rho_0]_c$ $(10^{-2}$ él. Å$^{-3})$	6,48	6,85	6,34	6,91	6,89
$\left[\frac{S_1}{v_1}\right]_c$ (Å^{-1})	0,157	0,174	0,161	0,180	0,184

On peut soulever plusieurs objections à cette interprétation des résultats expérimentaux.

(a) La forme de la molécule est, à pH 3,6, tellement anisodiamétrique, que la plus petite dimension (Tableau 4) n'est guère compatible avec la structure secondaire des chaînes polypeptidiques.

(b) On éprouve quelques difficultés à admettre que la structure de la protéine puisse être bouleversée aussi profondément par le changement du pH, sans que les modifications deviennent irréversibles.

(c) La variation du paramètre $(\rho_1 \quad \rho_0)$ avec la concentration est surprenante (voir ci-dessus); par ailleurs la différence entre les valeurs de $(\rho_1 - \rho_0)$ relatives aux deux conditions de pH, qui traduirait une grande différence du taux d'hydratation interne (qui passe de 0,34 g H_2O/g protéine à pH 5,3, à 1,15 à pH 3,6), est peu vraisemblable.

(d) Les propriétés hydrodynamiques de la SAB, interprétées en admettant que la particule a une forme compacte, ne sont compatibles qu'avec une forme sphérique, ou à peine anisodiamétrique, et ceci aux deux valeurs du pH (voir Tableau 4 et discussion plus loin).

On pourrait peut-être éviter ces écueils si on pouvait admettre que la polydispersité de masse ou de forme est plus grande à pH 3,6 qu'à pH 5,3: en fait les mesures de masse et les observations de Champagne (1957) sont en contradiction avec cette hypothèse.

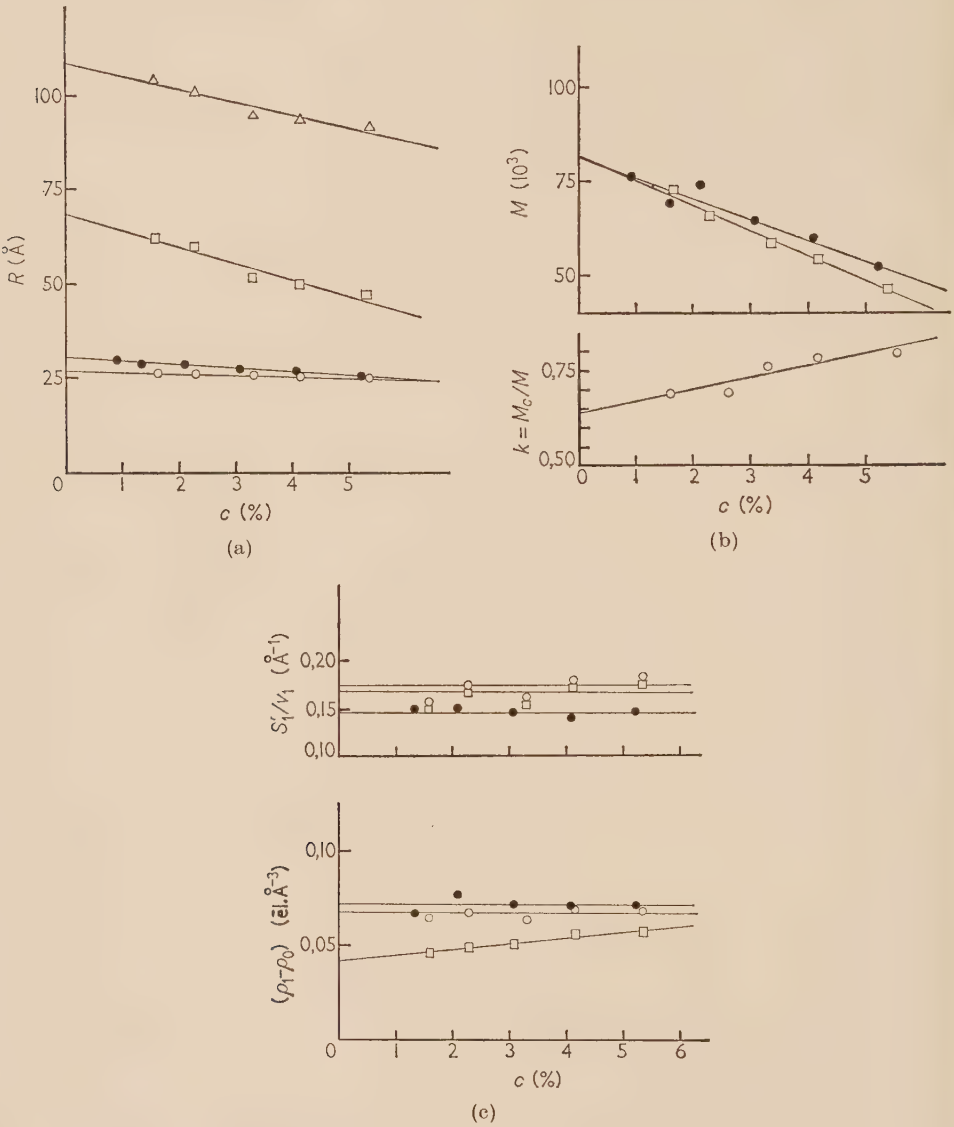


FIG. 3. Valeurs des différents paramètres, en fonction de la concentration, et droites obtenues par le calcul des moindres carrés.

● pH 5,3
□ pH 3,6, particule totale
○ pH 3,6, partie compacte
△ pH 3,6, partie lâche

C'est dans la structure de la molécule qu'il faut chercher une explication de ces anomalies. Or, s'il est évident d'une part, qu'il faut abandonner le modèle d'une particule compacte, d'autre part il convient de ne pas négliger la forme asymptotique des courbes

$j_n(s)$, qui suggère la présence d'un milieu à deux densités. Nous avons proposé il y a quelques années (Champagne *et al.*, 1958) un modèle qui satisfait à ces deux conditions: *à pH acide une partie de la chaîne polypeptidique se déroule, et adopte une configuration lâche, et probablement désordonnée, autour d'un noyau compact, formé par le reste de la molécule.* Le noyau compact, de petites dimensions, détermine la distribution de l'intensité diffusée aux angles relativement grands, et en particulier la forme asymptotique de $j_n(s)$: la contribution de la partie lâche, qui ne se manifeste qu'aux plus petits angles, est responsable de la brusque remontée de $j_n(s)$ qu'on observe près de l'origine.

Ce modèle est donc en bon accord avec la forme des courbes expérimentales: il se prête, en outre, à plusieurs vérifications quantitatives.

TABLEAU 3

Paramètres de la particule

	pH = 5,3	pH = 3,6		
		particule totale	partie compacte	partie lâche
M	81.200 (± 3.200)	81.700 (± 1.400)	52.200 (± 1.700)	29.500
R_0 (Å)	30,6 (± 0,4)	68,5 (± 2,5)	27,2 (± 0,4)	108,8
$(\rho_1 - \rho_0)$ (él. Å^{-3})	0,072 (± 0,002)	0,042 (± 0,001)	0,067 (± 0,004)	—
$\frac{S_1}{v_1}$ (Å^{-1})	0,146 (± 0,003)	0,17 (± 0,01)	0,175 (± 0,01)	—
v_1 (Å^3)	142.000 (± 7.000)	245.000 (± 9.000)	97.000 (± 7.000)	—
α	0,33 (0,34†)	1,10 (1,15†)	0,40 (0,42†)	

† gramme d'eau par gramme de protéine non hydratée.

Soient m_c et m_1, et $[R_0]_c$ et $[R_0]_1$ les nombres d'électrons et les rayons de giration de la partie compacte et de la partie lâche de la molécule, soit k la fraction des électrons de la molécule contenus dans la partie compacte

$$m = m_c + m_1 \tag{4}$$

$$m_c = km \tag{5}$$

$(c_e)_c = kc_e$ est la concentration électronique de la partie compacte. Si on admet que le volume spécifique ψ des deux parties est le même, on a pour s petit:

$$i_n(s) = c_e(1 - \rho_0\psi)^2 m \left[1 - \frac{4\pi^2}{3} R_0^2 s^2 + \dots\right] \tag{6}$$

$$[i_n(s)]_c = [c_e]_c\,(1 - \rho_0\psi)^2 m_c \left[1 - \frac{4\pi^2}{3}[R_0]_c^2 s^2 + \dots\right]$$

$$= k^2 c_e(1 - \rho_0\psi)^2 m \left[1 - \frac{4\pi^2}{3}[R_0]_c^2 s^2 + \dots.\right] \tag{7}$$

$$[i_n(0)]_c = k^2 i_n\,(0) \tag{8}$$

En outre, si on admet que les centres de gravité des deux parties coïncident:

$$R_0^2 = k[R_0]_c^2 + (1 - k)[R_0]_l^2 \tag{9}$$

Puisque l'intensité diffusée par la partie compacte devient prédominante aux angles relativement grands, on peut admettre, de manière plus précise, que $(j_n^*(s))_c$ coïncide avec $j_n^*(s)$ entre la région de s où la représentation de Guinier est linéaire (Fig. 2(b)) et l'infini, et que $(j_n^*(s))_c$ s'extrapole vers l'origine selon les droites de Fig. 2(b). On a ainsi:

$$[j_n^*(s)]_c = A \exp\left(-\frac{4\pi^2}{3}\alpha^2 s^2\right) + \phi(s) \tag{10}$$

$$[i_n(0)]_c = 2\sqrt{\frac{\pi}{3}}A\alpha - \frac{1}{\pi}\int_0^\infty s^{-2}\phi(s)\mathrm{d}s \tag{11}$$

$$[R_0]_c^2 = \frac{3}{4\pi^2}\left[\frac{8\pi^2}{3}\sqrt{\frac{\pi}{3}}A\alpha^3 + \frac{3}{2\pi}\int_0^\infty s^{-4}\phi(s)\mathrm{d}s\right] \times [i_n(0)]_c^{-1} \tag{12}$$

En connaissant $i_n(0)$, $[i_n(0)]_c$, R_0 et $[R_0]_c$ on peut calculer k (8) et $[R_0]_l$ (9). Les valeurs de ces paramètres sont portées dans le Tableau 3 et dans Fig. 3.

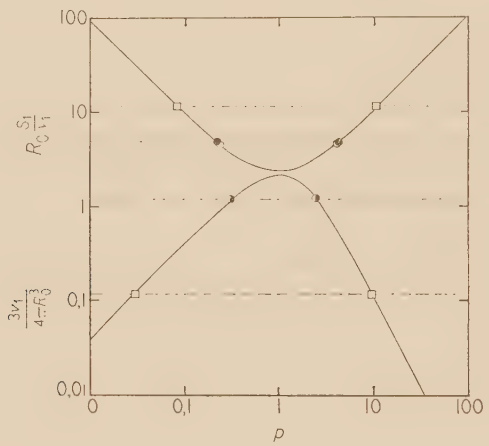


Fig. 4. Abaques relatifs aux ellipsoïdes de révolution:

● pH 5,3
□ pH 3,6, particule totale
○ pH 3,6, partie compacte

La variation de k avec la concentration (Fig. 3) s'explique plus facilement par un changement du rapport entre la masse des deux parties de la molécule que par un effet des corrélations. En effet la perturbation de $i_n(0)$, due aux corrélations, ne dépend que de la distribution des centres des molécules (Guinier & Fournet, 1955): si les centres de gravité de la partie lâche et de la partie compacte coïncident, $[i_n(0)]_c$ et $[i_n(0)]_l$ sont multipliés, à chaque concentration, par un même facteur, qui disparaît dans le rapport k.

S_1/v_1 également (Fig. 3) semble dépendre de la concentration: bien qu'en principe cette variation ne soit pas surprenante, puisque le rapport des masses dépend de la concentration, nous l'avons négligée, vu l'erreur qui affecte les mesures.

Les valeurs de M_c, M_l, $[R_0]_c$ et $[R_0]_l$, déterminées par une extrapolation linéaire à $c - 0$ (Fig. 3) sont portées dans le Tableau 3: la différence entre les dimensions des deux parties de la molécule est très grande.

On peut calculer les valeurs de $(\rho_1 - \rho_0)$ et de S_1/v_1, relatives à la partie compacte, en utilisant, dans les équations (LWN, éq. 11 et 12) la concentration kc_e de la partie compacte, au lieu de la concentration globale:

$$(\rho_1 - \rho_0)_c = \frac{\int_0^\infty 2\pi s[j_n^*(s)]_c ds}{kc_e(1 - \rho_0\psi)} + kc_e(1 - \rho_0\psi)\rho_0 \qquad (13)$$

$$\left(\frac{S_1}{v_1}\right)_c = \frac{16\pi^2 \lim_{s\to\infty}[s^3 j_n^*(s)]}{\int_0^\infty 2\pi s[j^*(s)]_c ds}\left[1 - \frac{k\rho_0 c_e(1 - \rho_0\psi)}{\rho_1 - \rho_0}\right] \qquad (14)$$

Il convient d'utiliser, dans (13) et (14), les valeurs de k relatives à chaque concentration.

Les valeurs de $(\rho_1 - \rho_0)$ (13) et de $(S_1/v_1)_c$ (14) sont portées dans le Tableau 2 et dans Fig. 3. Les anomalies de $(\rho_1 - \rho_0)$ ont disparu: d'une part, en effet, la variation avec la concentration devient négligeable, et d'autre part la valeur de $(\rho_1 - \rho_0)$ relative à la partie compacte est très voisine de celle de la molécule entière, au point isoionique.

On peut essayer de décrire la forme de la partie compacte par un ellipsoïde de révolution (Fig. 4 et Tableau 4): on obtient deux formes possibles, les deux à peine anisodiamétriques.

TABLEAU 4

Modèles d'ellipsoïdes

pH	$\frac{3v_1}{4\pi R_0^3}$	$R_0\frac{S_1}{v_1}$	p	a (Å)	b (Å)	D (10^{-7})	$[\eta]$	$D_{exp.}$ (10^{-7})†	$[\eta]_{exp.}$†
5,3	1,19		0,32	15,1	47,2	5,95	3,68		
			2,5	59,6	23,8	5,89	3,89	5,84	4,05
		4,47	0,24	11,4	47,7	5,63	4,42	6,07‡	3,88‡
			3,9	64,4	16,5	5,63	4,74		
3,6§	0,18		0,047	5,0	107,6	3,10	30,7		
			7,6	150	19,8	3,92	17,7	5,21	6,20
		11,5	0,085	9,0	106,5	3,64	16,6		
			10,7	152	14,2	3,48	28,0	5,42‡	6,94‡
3,6‖	1,20		0,33	13,8	41,9				
			2,5	53,0	21,2				
		4,76	0,22	9,4	42,5				
			4,3	57,9	13,4				

† Champagne, 1957
‡ valeurs corrigées pour la polydispersité (Champagne, 1957)
§ particule totale
‖ partie compacte

Les valeurs de D et $[\eta]$ ont été calculées à partir des valeurs de M et de v_1 données dans le Tableau 3, et des valeurs de p indiquées ici.

3. Discussion et Conclusions

On peut chercher dans d'autres travaux une confirmation de nos résultats expérimentaux, et une vérification du modèle du mécanisme de la dénaturation réversible.

Parmi les études de diffusion centrale des rayons X on peut citer celle de Anderegg, Beeman, Shulman & Kaesberg (1955), et celle, plus récente, de Echols & Anderegg (1960) : dans les deux cas les mesures ont été effectuées à une échelle relative. Anderegg *et al.* (1955) ont étudié la SAB à pH 4,6. Ils ont mesuré le rayon de giration ($R_0 = 29{,}8$ Å), et ils ont constaté que les courbes expérimentales pour $s < 25 \times 10^{-3}$ Å^{-1} sont en bon accord avec la courbe théorique relative à un ellipsoïde aplati ($p \simeq 0{,}3$); ils ont remarqué, toutefois, que les dimensions et la symétrie de la maille élémentaire des cristaux de SAB (Low, 1952) ne sont guère compatibles avec cet ellipsoïde, ce qui les a amenés à proposer une autre forme de particule. Ces auteurs ont en outre comparé les résultats des expériences de diffusion centrale avec ceux obtenus par des techniques hydrodynamiques; ils ont constaté que l'accord est satisfaisant si l'on admet que la particule occupe un volume $v_1 = 120.000$ Å^3, avec un taux d'hydratation interne de 0,37 grammes d'eau par gramme de protéine. Les résultats de ce travail sont en excellent accord avec les nôtres.

Echols & Anderegg (1960) ont étudié les modifications morphologiques de la SAB que provoque l'urée: ils ont mis en évidence une augmentation du rayon de giration (de 33,5 Å dans l'eau à 46,2 Å dans une solution d'urée 6 M) et des changements de la forme des courbes expérimentales qui suggèrent la présence d'un phénomène analogue à celui que nous décrivons ici.

Plusieurs auteurs ont déterminé la masse de la SAB aux deux conditions de nos expériences, et ils ont tous trouvé une seule valeur, indépendante du pH (Yang & Foster, 1954; Tanford, Buzzell, Rands & Swanson, 1955; Harrington, Johnson & Ottewill, 1956; Champagne, 1957). Les valeurs expérimentales de la masse moyenne, au point isoionique, vont de 70.000 à 84.000 (Champagne, 1957): la masse de la molécule est en fait voisine de 66.000 et c'est le composant lourd qui élève la valeur moyenne. La valeur que nous avons obtenue (81.000) correspondrait à une concentration de dimère de 20% environ, valeur qui semble un peu élevée (Champagne *et al.*, 1957). Notre mesure pourrait être affectée d'erreurs dues à une perturbation éventuelle de la distribution des ions au voisinage de la molécule (Luzzati, Nicolaieff & Masson, 1961), ou au choix du volume spécifique; il est au contraire peu probable que les coefficients d'absorption des filtres, ou tout autre erreur inhérente à la technique expérimentale, puissent justifier cet écart, puisque dans le cas du lysozyme (LWN) la valeur de la masse que nous avons obtenue est au contraire trop petite.

L'analyse la plus complète des propriétés hydrodynamiques de la SAB est celle de Champagne (1957): c'est surtout à ce travail que nous nous référons dans la suite (voir également Krause & O'Konski, 1959; Foster, 1960).

Au point isoionique la comparaison des valeurs de la masse, de la viscosité intrinsèque, du coefficient de diffusion de translation et de la constante de sédimentation permet à Champagne de montrer que, si la SAB est une particule rigide, sa forme peut être assimilée à une sphère, ou éventuellement à un ellipsoïde faiblement anisodiamétrique. Ceci est confirmé par l'accord entre les valeurs théoriques de la viscosité intrinsèque et du coefficient de diffusion de translation, calculées pour les différents ellipsoïdes compatibles avec nos mesures (LWN éq. 18 et 19), et les valeurs expérimentales (Tableau 4).

A pH 3,6 tous les auteurs ont constaté que le volume hydrodynamique augmente: bien que certains aient proposé une forme anisodiamétrique (Harrington *et al.*, 1956), les mesures les plus récentes et les plus précises (Yang & Foster, 1954; Champagne, 1957) indiquent en fait que la particule ne peut avoir qu'une forme sphérique, si on admet qu'elle est rigide. Certains auteurs (Yang & Foster, 1954; Tanford *et al.*, 1955) estiment que l'augmentation du volume est due à un gonflement isotrope, sans dépliement de la chaîne polypeptidique; au contraire les expériences de pouvoir rotatoire (Jirgensons, 1958) indiquent que les changements qu'on observe entre pH 3 et 4 sont dûs à des modifications de la structure interne.

Nous avons comparé (Tableau 4) les valeurs de $[\eta]$ et D correspondant aux ellipsoïdes compatibles avec nos mesures, et les valeurs expérimentales: le désaccord est flagrant, surtout par contraste avec la SAB au point isoionique et avec le lysozyme. Il est donc évident qu'à pH 3,6 la SAB n'est ni compacte ni rigide.

Le modèle de structure, à pH 3,6, que nous avons proposé et analysé ci-dessus n'est évidemment pas le seul qui soit compatible avec nos résultats expérimentaux. Ainsi on pourrait admettre que la partie compacte, au lieu de former un seul noyau, est partagée en plusieurs fragments liés entre eux. De même la chaîne polypeptidique dépliée qui forme la partie lâche pourrait être formée de plusieurs segments ou boucles: toutefois le nombre de ces filaments ne peut pas être grand, et la structure doit être très lâche, pour que le rayon de giration atteigne 109 Å, avec une masse $M = 29.500$.

Le modèle discuté ici est, dans ses grandes lignes, celui que nous avons proposé en 1957 (Champagne *et al.*). Dans nos conditions expérimentales d'alors nous ne pouvions pas enregistrer l'intensité diffusée au dessous de $s = 6.10^{-3}$ Å^{-1}, et nous n'avions pas pu observer la remontée de l'intensité au voisinage de l'origine: le modèle, toutefois, en postulait l'existence. Les dimensions obtenues en 1957 à pH 5,1 et 3,6 (ces dernières se réfèrent à la partie compacte) sont en assez bon accord avec les mesures récentes (Tableau

pH	R_0(Å)	v_1(Å^3)	$\frac{S_1}{v_1}$ (Å^{-1})
5,1	31,5	130.000	0,160
3,6	30,5	90.000	0,200

3), quoique certaines différences dépassent l'écart qu'on peut attribuer aux erreurs expérimentales. Ces désaccords peuvent tenir aux différences entre lots de SAB (voir Echols & Anderegg, 1960), et également au fait que nous avons délipidé la protéine au cours des dernières expériences (voir partie expérimentale), ce que nous n'avions pas fait précédemment.

Il convient de remarquer que nous avons négligé, dans notre interprétation, la polydispersité de l'échantillon: les valeurs des différents paramètres sont en fait des valeurs moyennes (Guinier & Fournet, 1955). La polydispersité affecte la précision des résultats, sans compromettre, toutefois, l'essentiel des conclusions.

Nous avons souvent bénéficié au cours de notre travail des conseils et des critiques de M. H. Benoit et de Mlle M. Champagne: qu'ils veuillent bien trouver ici l'expression de notre reconnaissance.

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Densities of Transducing Lambda Bacteriophages

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Phages of a lysate of an induced culture of bacteria lysogenic for λ when centrifuged in a CsCl density gradient form a narrow band having a maximum at a definite density. The rare galactose-transducing phages contained in such a lysate are shown here to form a much wider band (especially towards the lower densities) having no definite maximum. Each transducing phage selected at a given density, when allowed to multiply, is shown to form a density band as narrow as that of λ but with a maximum at the selected density. The transducing phages are also shown to be defective. It is concluded that these transducing phages have acquired their density and their defect when they were first produced and not during their subsequent transformation into prophages.

1. Introduction

Bacteria lysogenic for the phage λ, when induced, yield lysates which contain a small proportion of phages able to transduce to other bacteria the galactose markers carried by the lysogenic cells. These lysates are called LFT (low frequency transducing).

Most of the bacteria in which the galactose markers have been transduced carry, in addition to the prophage of transducing λ, two sets of galactose markers: their own and those brought in by the transducing phages. At the multiplicities of infection usually employed for transduction with LFT lysates, all the bacteria are also infected with non-transducing phages. The transduced bacteria are thus doubly lysogenic. Upon induction they yield plaque-forming and transducing phages in approximately equal numbers; these lysates are called HFT (high frequency transducing).

The transducing phages, called *λdg*, in these HFT lysates have been studied in detail (Arber, 1958). They are defective apparently because they carry a deletion (Campbell, 1959) but the defect can be overcome (complementation) by the presence in the infected cell of a plaque forming "helper" λ. The helper λ also increases twenty- to thirty-fold the lysogenization and the transduction by *λdg*.

The buoyant densities, in CsCl solutions, of *λdg* phages have been measured (Weigle, Meselson & Paigen, 1960). They are, in general, different for each *λdg* of independent origin and may be smaller or larger than the density of λ. The density of *λdg* is transmitted to its offspring.

In contrast to these studies of *λdg* from HFT lysates, practically nothing is known about the transducing phages contained in LFT lysates except that after transduction they give rise to *λdg*. Their densities are unknown and their defectiveness not

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established. It is possible that, before their first lysogenization-transduction and their transformation into *λdg* prophages, they had the density of λ and no defect.

It will be shown below that their densities are altered and stably established before their transformation into HFT *λdg* and that they are defective.

2. Material and Methods

Bacteria. 1. Lederberg's strain 3110 F^-, prototroph, *Gal*$^+$, lysogenic for λ called *Gal* 1^+2^+ (λ).

2. A strain derived from 3110 into which the markers *Gal* 1^- and *Gal* 2^- have been introduced by transduction. This strain is called *Gal* 1^-2^- when it is sensitive and *Gal* 1^-2^- (λ) when it is lysogenic.

3. Different heterogenotes *Gal* 1^-2^- (λ) (*λdg Gal* 1^+2^+) lysogenic for λ and *λdg*, as described in the text.

Phages. λ wild type (Kaiser, 1957) called here simply λ. *λcm6* (Jacob & Wollman, 1954) used here as a density reference marker.

The techniques employed have been described previously (Weigle *et al.*, 1960).

3. Results

(a) *Density distribution of transducing phages*

If the transducing phages in an LFT lysate have not been altered in density we should find them where we find λ in a density gradient centrifugation. The "band" of the transducing phages should be superposed on the band of λ.

A typical density gradient centrifugation proceeded as follows. A culture of *Gal* 1^+2^+ (λ) was induced with a small dose of u.v. (inducing approximately 80% of the bacteria). The lysate was purified by centrifugation and the phages were resuspended in 3·7 ml. of a solution of CsCl with a final density of 1·50 g/ml. The suspension containing $4{\cdot}6 \times 10^{11}$ phages was transferred to a plastic tube in a swinging bucket rotor of a Spinco preparative centrifuge. After spinning for 14 hr at 27,000 rev/min the tube was removed and a small hole pierced in the bottom. The band of phages was visible and its slow motion downwards could be observed as the emerging drops were being collected in separate tubes. The contents of each tube were assayed for plaque forming and for transducing phages. For the latter assay *Gal* 1^-2^- sensitive bacteria were infected simultaneously with the phages to be tested and with 4 helper phages per bacterium. These helper phages had been obtained from an induced culture of *Gal* 1^-2^- (λ). In each experiment controls were made with *Gal* 1^-2^- sensitive bacteria infected with helper phages only. These controls never showed *Gal*$^+$ bacteria. The results are represented in Fig. 1. One sees that the distribution in density of the transducing phages is very wide and very irregular while that of the plaque formers is narrow and approximately Gaussian. Different independent LFT lysates, obtained from cultures of *Gal* 1^+2^+ (λ) started from single colonies gave approximately the same number of transducing phages with a similar range in the density distribution of these phages. The details of the distribution were however very different. The fairly wide fluctuations in the number of transducing phages in different drops as well as the different distribution in different lysates could be due to the presence of large clones of transducing phages of uniform density.

The conclusion to be drawn from these experiments is that the transducing phages contained in LFT lysates are not uniform in density. Their densities are widely and

asymmetrically distributed about the uniform density of the plaque forming phages. The results of the next section show that the densities of the transducing phages are transmitted to their offspring.

(b) *Genetic stability of the density of the transducing LFT phages*

This section describes experiments designed to measure the density of the progeny of the transducing LFT phages and to study the correlation between this density and the density of the drop in which the LFT phages appear.

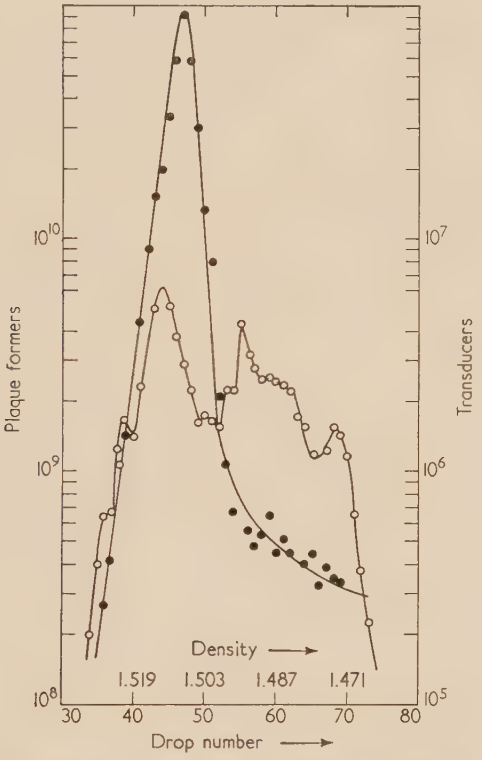

FIG. 1. Titers of the plaque-forming phages (filled circles) and of the phages transducing the *Gal* 1^+ and the *Gal* 2^+ markers together (empty circles) in the drops collected after density gradient centrifugation. In abscissa, the drop number and its calculated density; in ordinates, the logarithm of the titers.

Each drop, collected after the centrifugation, comes from a definite layer of liquid in the centrifugation tube. Since a gradient of density has been formed by the centrifugation each drop has a characteristic density. In Fig. 1 the values of the densities are shown along the abscissa. These values have been calculated from the mean density, the speed of the rotor and the distance of the liquid from the axis of rotation.

The width of a band of phages in the CsCl gradient is due to the Brownian motion of the particles and to any inhomogeneity in density they may show. Thus a layer of liquid contains phages with buoyant densities which differ from that of the layer, these phages having diffused to that layer by Brownian motion. If the phages do not have a uniform density, as in the case of the transducing LFT phages, each drop

collected from the centrifugation tube contains a population of phages whose densities are distributed about the density of the drop.

The phages contained in a drop of an LFT lysate collected after centrifugation were used to transduce the *Gal*$^+$ markers to *Gal*$^-$ sensitive bacteria. With the help of plaque forming phages they produced heterogenote *Gal*$^-$ (λ) (λdg *Gal*$^+$) bacteria. These heterogenotic strains, when induced yield λ and λdg. If the transducing LFT phages transmit their density to the λdg's, a strong correlation should be found between the densities of the λdg's and the drop number since the number of the drop is a measure of its density.

The experiments were done as follows. *Gal* 1 2$^-$ sensitive bacteria were transformed into *Gal*$^+$ bacteria by the transducing phages of drops 30, 38, 48, 60 and 70 of Fig. 1. For these transductions a helper phage obtained from the induction of *Gal* 1–2$^-$ (λ) was used at a multiplicity of 4 phages per bacterium. The transformed bacteria were picked and twice purified by streaking. Cultures were then grown from single colonies, induced, and the resulting HFT lysates were diluted in a CsCl solution of the proper density. A convenient fraction of the suspension of a phage ($\lambda cm6$) used as a density reference was added to the different preparations. They were then put, one at a time, into the cell of an analytical centrifuge. After spinning at 27,690 rev/min for 12 hr the distribution of the phages in the cell was photographed by u.v. absorption. The densities of the different λdg phages were calculated from the distances between their bands and that formed by the reference material. They are given in Table 1.

TABLE 1

Buoyant densities in g/ml. of the λdg *phages obtained from heterogenotes formed by the transducing LFT phages described in Fig. 1*

Usually more than one heterogenote was picked from the transductions of each collected drop and given an "isolate number".

Drop number	Calculated density of the drop	Isolate	Density
70	1·471	4	1·487
70	1·471	1	1·491
60	1·487	1	1·502
48	1·506	1	1·505
48	1·506	2	1·508
38	1·522	1	1·508
38	1·522	0	1·511
38	1·522	4	1·512
30	1·535	4	1·515
30	1·535	1	1·517

It can be seen that each heterogenotic strain λdg of a characteristic density, in general different from that of λ whose value is 1·508 g/ml. The λdg phages are the denser the lower the drop number, that is, the greater the density from which the parental transducing phage was selected. One may thus conclude that the transducing LFT phages transmit their densities to the λdg derived from them. In other words the density is a stable heritable character of an LFT transducing phage.

(c) *The defectiveness of transducing LFT phages*

The overall proportion of transducing phages, among the plaque formers of an LFT lysate, is so small that it is experimentally difficult to decide whether or not they are defective. In view of this small proportion most of the bacteria transformed with an LFT lysate contain also the prophage of plaque forming λ which came in through simultaneous infection or through superinfection on the plates. The presence of this prophage may thus mask the defectiveness of the transducing prophage.

As can be seen in Fig. 1 the drop no. 56 contained approximately $5{\cdot}8\times10^{8}\lambda$ and $3{\cdot}2\times10^{5}$ effectively transducing phages. If we assume with Arber (1958) that with helper phages the efficiency of transduction is 0·20 the drop 56 contained $1{\cdot}6\times10^{6}$ transducing phages. The proportion of transducers among plaque formers being thus $2{\cdot}8\times10^{-3}$ makes it feasible to detect defective heterogenotes and to measure the increase in the efficiency of transduction by helper phages.

Gal 1-2 sensitive bacteria were infected at very low multiplicity ($\sim 10^{-3}$ plaque formers per bacterium) with the phages of drop 56. They were then plated with concentrated anti-λ serum (to avoid as much as possible re-infection on the plates). On 10 plates the average number of transformed bacteria was 2 per plate at the dilution used. *Gal* 1 2- bacteria infected in the same fashion with the phages of drop 56 but superinfected with 4 helper phages per bacterium yielded approximately 20 times more transformed bacteria (40 and 36 respectively on 2 plates). Thus the helper phages increase the efficiency of transduction by a factor of about 20.

The 20 colonies transformed without helper phage, were picked and twice purified by streaking. All but two segregated *Gal*- colonies, 18 were thus heterogenotes. Of these, 10 were defective and 8 yielded both λ and λ*dg* upon induction. With the multiplicity employed of about 10^{-3} phage per bacterium 4 cells were expected to have been simultaneously infected with λ and λ*dg*. Thus a large proportion if not all of the transducing phages in LFT lysates are defective in the sense that upon lysogenization of a sensitive bacterium they yielded defective heterogenotic strains.

4. Discussion and Conclusions

The experiments described above show that the transducing phages contained in an LFT lysate are altered in density and that they transmit their density to the λ*dg* phages of the HFT lysates that can be derived from them. Their first act of transducing is thus not responsible for the density alteration. Since at least a large fraction of these LFT transducing phages are defective it is tempting to think that they are identical to the λ*dg* phages in HFT lysates.

The heterogeneity in density found among the transducing phages is remarkable: relatively few have densities larger than that of λ while many more have smaller densities; besides, the amplitude of the variation in density is about twice as large for the phages of the smaller densities than for those of the larger ones.

A simple model can account for these facts. Let us first assume that the alterations in density are due only to the DNA content of the phages. Then, since the transducing phages are defective because they have lost part of their genome (Arber, 1958; Campbell, 1959), let us suppose that this loss is due to a translocation in which part of the genome of the phage is deleted and replaced by a part of the bacterial genome comprising the *Gal* markers. Since, according to Morse, Lederberg & Lederberg

(1956), the transducing phages contain all the *Gal* genes of the donor bacterium we may assume that the piece of bacterial genome introduced into the phage is always the same size. If we now suppose that the size of the deletion may have any value, between almost zero and a certain maximum, the transducing phages would have the observed heterogeneity in density. The most dense phages would carry the smallest deletion and the least dense the largest.

These rather oversimplified assumptions allow determinations of lower limits for the amount of DNA involved in the translocation. One can calculate the fractional increment α of DNA necessary to produce an increase $\Delta\rho$ in density from the relation (see Weigle *et al.*, 1960)

$$\Delta\rho = 0{\cdot}10\alpha$$

For the densest phage (drop 30, isolate 4 of Table 1) one finds $\alpha = 0{\cdot}075$. If we assume that for the phages of this drop the size of the deletion is negligible, a lower limit for the size of the bacterial genome involved in the translocation represents 7·5% of the DNA contained in plaque forming λ. The density of the least dense phage gives by a similar calculation a lower limit for the size of the maximum deletion: it amounts to 28% of the DNA of λ.

The presence of a density determining genetic marker close to and perhaps involved in the deletion (Kellenberger, Zichichi & Weigle, 1960) may bring complications into this simple model. This density marker may be responsible for the lack of complete correlation between densities and the loss of genetic material observed by Campbell (1959).

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A Mutation Affecting the DNA Content of Bacteriophage Lambda and its Lysogenizing Properties

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A mutation of phage lambda conferring on the phage a buoyant density lower than that of the wild type is described. The genetic character responsible for this alteration is located in the region *m*5-*h* of the lambda chromosome. The phage *λb*2 contains about 18% less DNA than the wild type. The *λb*2 mutant does not multiply as prophage, but establishes abortive lysogeny.

1. Introduction

Mutations are known which affect the density of bacteriophage *λ* (Kellenberger, Zichichi & Weigle, 1960). The genetic aspects of one of these are described. It is shown that the density is controlled by a genetic marker called *b*2 located on the genetic map of *λ* in the region *m*5 to *h*. This genetic marker behaves in crosses like any other known marker of *λ*.

The phages *λb*2$^+$ (wild type of Kaiser, 1957) provoke either a lytic or a stable lysogenic response in the bacteria they infect. The phages *λb*2, having a smaller density than *λb*2$^+$ either lyse the bacteria or establish an abortive lysogenization; that is, the prophage can remain in the bacteria without multiplying while the bacteria divide. During the growth of such bacteria the prophage is given to one, only, of the daughters, which then remains inducible to lysis with u.v. irradiation.

The alteration in density has been found to be due to the amount of DNA contained in the phages. The *λb*2$^+$ particles have an excess of DNA which amounts to about 18% of the DNA of *λb*2.

The implications of these findings are discussed.

2. Materials and Methods

Bacterial strains derived from Escherichia coli *K12*. W3110 prototroph *λ* sensitive (Lederberg); C600 (Appleyard, 1954) and CR63 (Appleyard, MacGregor & Baird, 1956).

Bacteriophages. *λ* wild type § (Kaiser, 1957) and some of its mutants *λc mi*, *λc m*6, *λm*5, *λh*, or recombinants between these, have been used.

† Aided by a grant from the Swiss National Foundation for Scientific Research.
‡ Aided by a grant from the National Foundation.

§ In two previous publications (Weigle *et al.*, 1959 and Kellenberger *et al.*, 1960) this wild type has been called by mistake Kaiser's reference type. Kaiser's (1955) reference type probably carries the density marker *b*2 since it does not produce stable lysogenization. This reference type was crossed by Kaiser with a phage producing stable lysogenization used by Jacob & Wollman (1954) to obtain what he has called "wild type".

Media. Tryptone broth (1% Difco Tryptone with 0·5% NaCl) or M9 (with 1% Difco Casamino acids added) were used as growth media. Adsorption medium: 0·01 M-$MgSO_4$ in distilled water. Solid media: Tryptone broth with 1·5% agar.

The methods used for plating have been described by Adams (1950). Analytical as well as preparative density-gradient centrifugations were conducted as described in Weigle, Meselson & Paigen (1959).

3. Results

A mixture, in approximately equal quantities, of $\lambda + +$ (wild type) and $\lambda m6\ c$ when centrifuged in a CsCl gradient in the analytical centrifuge produces two well-separated bands as shown in Plate I. The photometer tracing of the photograph of Plate I is shown in Fig. 1. It can be seen that the two bands are of the same width.

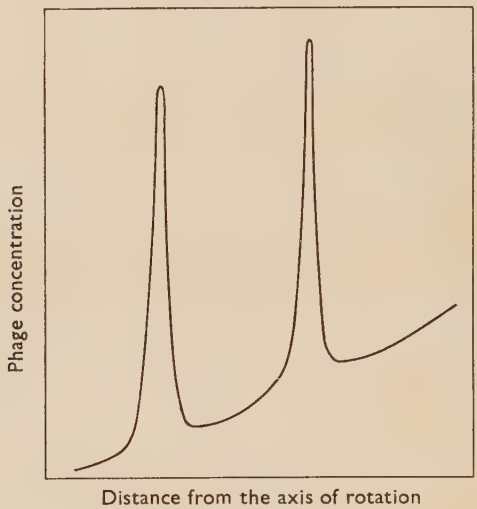

FIG. 1. Photometric tracing of Plate I.

If the mixture is centrifuged in the swinging bucket rotor of the preparative centrifuge and drops collected the $\lambda + +$ is found in the early (denser) drops and $\lambda m6\ c$ in the later drops. Thus $\lambda + +$ is denser than $\lambda m6\ c$. Small perturbations in the flow of the drops may produce slight changes in the shape and the width of the bands observed, but usually less than 0·1% of the phages of one type are found in the band of the other type. A knowledge of the initial density of the phage suspension in CsCl and of the speed of the centrifuge is used to calculate the two densities. They are found to be 1·508 g/cm^{-3} for $\lambda + +$ and 1·491 g/cm^{-3} for $\lambda m6\ c$ (and also for phage $\lambda c\ mi$). We shall call these B_0 and B_2 respectively.

(a) *Description of a typical cross*

A mixture of equal numbers of $\lambda + +$ and $\lambda m6\ c$ is adsorbed onto W3110 bacteria starved in adsorption medium with a multiplicity of 4·5 phages of each type per bacterium. After 15 min allowed for adsorption (99·5% of the input), the bacteria are diluted in Tryptone and the suspension is aerated at 37°C for 75 min and then chloroformed. To analyse the progeny of the cross by density gradient centrifugation a small portion of the lysate is added to a CsCl solution of the appropriate density in the plastic tubes of the swinging bucket rotor. After centrifuging for 14 to 16 hr at

PLATE I. Photograph by u.v.-absorption of the centrifuge cell after sedimentation equilibrium has been approached (27,690 rev/min at 20°C for 14 hr). The initial density of the suspension was 1,500 g/cm^{-3} and it contained $\lambda + +$ (left band) and $\lambda m6\ c$ (right band).

[*To face page* 400

22,000 rev/min the tubes are removed, a small hole pierced in the bottom of each one and the emerging drops collected in separate tubes containing 1 ml. of Tryptone. Each drop is then analysed for the number of phages it contains and for the genetic characters of these phages. The results of this analysis are given in Fig. 2. All the progeny phages have the density of one or other of the parents; there are practically

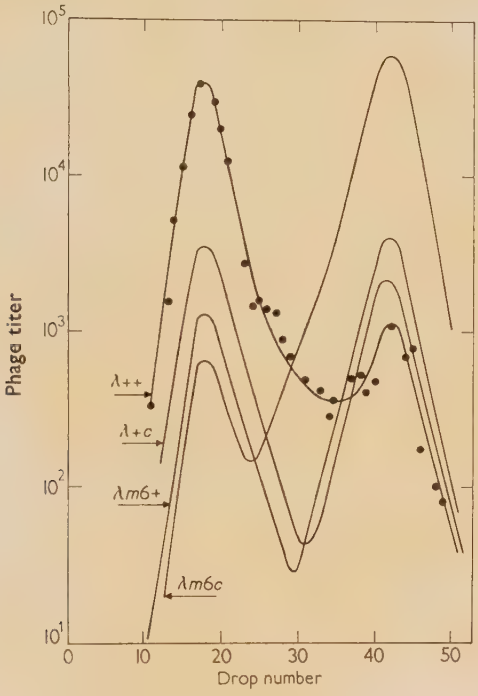

FIG. 2. The progeny of the cross $\lambda + + b2^+ \times \lambda m6\ c\ b2$ was centrifuged in a lusteroid tube of the swinging bucket rotor of the preparative centrifuge (22,000 rev/min for 14 hr). The plastic tube was removed, its bottom pierced with a needle and drops collected for subsequent examination. The log of the titer of the phages of each genetic type in each drop is the ordinate (arbitrary units) while the drop number is the abscissa. To give an idea of the accuracy of the method without crowding the figure the experimental points are shown only for $\lambda + +$; the scattering of the points around the smoothly drawn curves was practically the same for all types of phage.

no phages of intermediate densities. The band of high density contains mostly the parent $\lambda + +$ and the low density band mostly the parent $\lambda m6\ c$. However, there appear, as a result of the cross, a considerable number of phages having the parental morphological markers which have changed their densities. The recombinants $\lambda + c$ and $\lambda m6 +$ are also distributed unequally among the two densities.

(b) *The densities are hereditary characters*

To show that the densities acquired by the phages in a cross between phages of different densities are genetic characters it is necessary to prove that the offspring of these phages have the same density as their parents. Hence phages were picked in the central drop of each band, a stock of each phage was prepared by multiplication on W3110 and it was then centrifuged in the CsCl gradient with one of the two parents as a density reference. First phages were selected which had the parental morphological markers but which were of changed density (for example $\lambda + +$ of

density B_2 and λ *m6 c* of density B_0 of the cross described in Fig. 2). Then the recombinants $\lambda m6$ +, λ + *c* of both densities (or λc +, λ + *mi* in the cross λ + + $B_0 \times$ λc *mi* B_2) were studied. The progeny of 20 phages were studied in this way. All maintained the density of the band from which the original phage had been selected.

Crosses of the type $B_0 \times B_0$ or $B_2 \times B_2$, no matter what morphological markers the phages carry, give progeny phages of one density only: that of the parents. Crosses $B_0 \times B_2$ always give two bands of the densities B_0 and B_2 with the recombinants distributed in the two bands as in Fig. 2. The density B_2 thus behaves as a single genetic character independent of the morphological characters of the phage. We shall call this genetic character *b2* and its allele in λ wild type $b2^+$ giving the phenotype B_0.

To test whether phenotypic mixing occurs in a cross $B_0 \times B_2$ all the phages contained in the central drop of the bands B_0 and B_2 respectively were adsorbed on W3110 bacteria at very low multiplicity. After one cycle of multiplication, to allow the genotype to be expressed, the two lysates were again centrifuged in the CsCl gradient. Each of the lysates contained one band only and this band appeared at the density at which it had been selected. Less than 1% of the total phages of the lysates appeared in the drops which should have contained phages of the other density. One concludes then that there is no phenotypic mixing, that is, phage of the phenotype B_0 have the genotype $b2^+$ and phage of the phenotype B_2 have the genotype *b2*.

(c) *Localization of* b2 *on the genetic map of* λ

Genetic crosses with the density markers *b2* and $b2^+$ do not yield accurate results for two reasons. First the presence of *b2* may confer to the phage harboring this marker a definite advantage. Secondly the separation of the densities of the CsCl gradient centrifugation is not perfect: a band is always followed by a tail which may spread into the next band.

TABLE 1

Crosses relating the position of b2 *to the position of the markers* c, mi, m6, m5 *and* h

Parents	+ + +	*b2 c mi*	+ *c mi*	*b2* + +	+ + *mi*	*b2 c* +	+ *c* +	*b2* + *mi*
+ + + × *b2 c mi*	5476	8420	533	910	198	445	104	140
	+ + +	*m6 b2 c*	+ *b2 c*	*m6* + +	+ + *c*	*m6 b2* +	+ *b2* +	*m6* + *c*
+ + + × *m6 b2 c*	5325	9647	311	177	531	587	172	111
	. *m5* +	*m6* + *b2*	+ + *b2*	(*m6m5*+)	*m6* + +	. *m5 b2*	(*m6m5b2*)	+ + +
+ *m5* + × *m6* + *b2* †‡	3137	4074	190	—	27	38	—	8
	+ + *c*	*h b2* +	*h* + *c*	+ *b2* +	+ + +	*h b2 c*	+ *b2 c*	*h* + +
+ + *c* × *h b2* +	3823	4565	26	57	335	315	(150)§	17

Eight central drops of each peak of density B_0 and B_2 respectively were pooled. After convenient dilutions the phages and their genetic markers were determined with the results given above.

† The phages *m6 m5* + cannot be distinguished from + *m5* +.

‡ The phages *m6 m5 b2* cannot be distinguished from + *m5 b2*.

§ These phages are in the trailing end of the high peak of + + *c* and they may be mostly of this type.

Nevertheless the data of three factor crosses given in Table 1 shows clearly that *b*2 is not between *c* and *mi* but is between *c* and *m*6. The other two crosses involving *m*5 and *h* show that *b*2 is close to both of them. If the numbers of Table 1 were to be taken without correction one would find the genetic map given in Fig. 3.

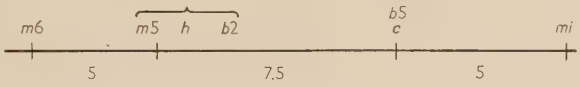

Fig. 3. Genetic map of λ.

(d) *Abortive lysogenization of the phages carrying the marker* b2

To measure the frequency of lysogenization of the phages these were mixed with bacteria W3110 prepared for adsorption. After 20 min at 37°C the bacteria were spread on plates coated with anti-λ serum (0·1 ml. per plate of a serum having a K of 25/min). After 12 hr incubation at 37°C the colonies were replicated onto plates seeded with C600 bacteria. These were irradiated with u.v. to induce lysogenic bacteria. After incubation the replica plates showed the lysogenic colonies surrounded by a halo due to the lysis of C600 by the liberated phages, while the non-lysogenic colonies had no halo. The experiments were usually made at a multiplicity of infection of 0·3 phages per bacterium and about 5000 bacteria were analysed for each type of phage. The phage $\lambda b2^+c^+$ gave usually 100 to 200 stable lysogenic colonies while $\lambda b2\ c^+$ showed 2 to 8 colonies surrounded by a halo. These were unstable lysogenic as shown by the following data: When a colony apparently lysogenic for λ*b*2 is picked and the bacteria spread again on anti-λ serum plates one finds that about 90% of the resulting colonies are not lysogenic but sensitive, the remaining 10% being again unstable segregating about 90% of sensitive colonies. This type of behaviour has already been described by Jacob & Wollman (1954) for the phages carrying a marker "*l*" located in the region of *m*5. They called this type of response abortive lysogenization in analogy with abortive transduction. The density marker *b*2 may be identical to "*l*". The phage λ*c mi* and λ*m*6 *c* which were found to have the marker *b*2, may however have carried also the marker "*l*" and the change in lysogenizing properties might have been due to "*l*" rather than to *b*2. In order to show that *b*2 was responsible for the abortive lysogenization we tried to isolate other *b*2 mutants.

In principle the experiments are carried out as follows: A lysate of $\lambda b2^+$ (obtained either by induction of a lysogenic strain of bacteria or by infection and subsequent lysis of a sensitive strain) is centrifuged in CsCl. The drops expected to contain phages of density B_2 are again centrifuged to eliminate contaminating $\lambda b2^+$, and the process is repeated. The phage are then passed through one cycle of multiplication on W3110 bacteria. One finds in this way that a stock of $\lambda b2^+$ contains between 10^{-6} to 10^{-7} of genotype *b*2. This measurement is made difficult because of the presence of a number of phages of the phenotype B_2 having the genotype $b2^+$. About 1 in 10^4 phages of the original lysate has this property. The meaning of this phenomenon is unknown. Besides, among the λ*b*2 isolated in this fashion quite a few carry morphological markers which the parent $\lambda b2^+$ did not have so that we do not know whether the λ*b*2 morphologically similar to the parent $\lambda b2^+$ are isogenic or not. Five *b*2 mutants from 5 different lysates obtained by this procedure were studied. They all produced abortive lysogenization, which makes us believe that abortive lysogenization is due to the marker *b*2.

(e) *Analysis of abortive lysogenization*

Bacteria infected with more than about five $\lambda b2^+c^+$ do not produce phages in liquid medium (Fry & Gros, 1959.) Most of them become lysogenic. In our experimental conditions non-infected starved bacteria when placed in nutrient medium start dividing and resume normal growth after a lag period of one hour. When infected with five $\lambda b2^+c^+$, this lag period is increased by about 20 min and the growing bacteria are all lysogenic. A variable proportion (5 to 40%) of the cells however do not survive the infection either as infective centers or as lysogenic colony formers; this proportion depends upon the multiplicity of infection and upon the physiological state of the bacteria at the time of infection.

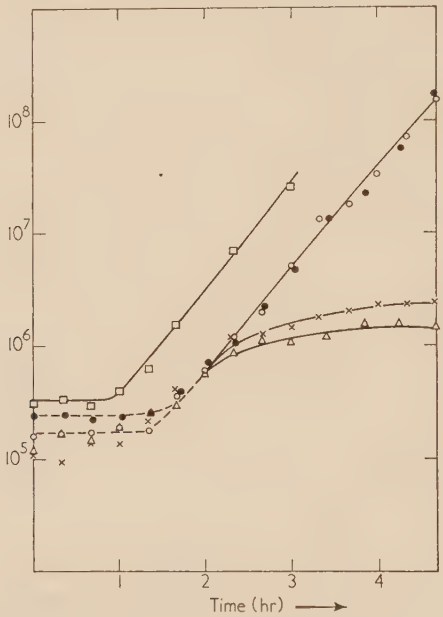

FIG. 4. Starved W3110 bacteria were infected with $\lambda b2$ *c* at multiplicities of 5 and 10 phages per bacterium. A sample of uninfected bacteria was used as control. After adsorption the three types of bacteria were diluted at time zero in Tryptone broth containing antiserum (K = 25/min). At the different times given in the abscissa the bacteria were plated to measure the colony formers. Samples of the infected bacteria were also given an induction dose of u.v. and plated on C600 to measure the number of phage-yielding bacteria.

□ uninfected bacteria
● infected bacteria m.i. = 5† } colony formers
○ infected bacteria m.i. = 10 }
△ m.i. = 5
× m.i. = 10 } phage-yielding bacteria after induction

† multiplicity of infection.

Infection with five $\lambda b2\ c^+$ (or more) phages per bacterium produces the same effect: 95% of the bacteria do not lyse and after transfer to Tryptone broth at 37°C a large proportion of them start dividing again after a lag of about 80 min (Fig. 4). During the first hour 30 to 50% of these bacteria are inducible: they lyse and liberate phages after u.v. irradiation.† After 80 min the number of inducible bacteria is equal

† The number of inducible bacteria during the first hour is smaller than the number of surviving bacteria for it is known that only about 30% of *starved* bacteria can be induced with the "normal doses" of u.v.

to the number of surviving bacteria. Later, as the bacteria divide, the proportion of productive bacteria after induction decreases. The number of productive bacteria is never larger than the number of infecting phages. It seems that the infecting phages are distributed, without multiplying, among the daughter bacteria. Indeed after a certain length of time the number of productive bacteria remains constant while the total number of bacteria continues to increase and a larger and larger proportion of bacteria become sensitive to λ and do not lyse after u.v. induction.

It thus seems that the prophages *b*2 persist in the bacteria infected at high multiplicity; they do not divide and do not prevent the division of the bacteria. The analogy with abortive transduction is complete and the term abortive lysogenization seems thus justified.†

It may be mentioned that after u.v. irradiation of abortive lysogenic bacteria the lysates do not contain any transducing phages. This indicates that the relations of the abortive prophage to the bacterial chromosome, if any, are quite different from those of the normal prophage.

(f) *DNA content of* λb2$^+$ *and* λb2

The difference in density of the phages *b*2 and *b*2$^+$ could result from changes in the intrinsic densities and relative amounts of DNA, protein or other possible viral constituents or from changes in packing or solvation. None of these possibilities is exclusive of the other. It has been shown by Weigle *et al.* (1959) that if the amount of DNA alone is responsible for the alteration in density, and if $B_0 - B_2 = \Delta\rho$, then

$$\Delta\rho = \frac{0{\cdot}21\alpha}{2+\alpha} \qquad (1)$$

where α is the fractional increase in DNA content of *b*2$^+$. With $\Delta\rho = 1{\cdot}508 - 1{\cdot}491 = 0{\cdot}017$, $\alpha = 0{\cdot}18$, i.e. *b*2$^+$ would contain 18% more DNA than *b*2. This should be observable if the DNA content per particle is measured.

Accordingly two stocks of the phage $\lambda b2^+c$ and $\lambda b2\ c$ were prepared. These were purified and concentrated by cycles of high and low speed centrifugation and after being mixed in equal quantities in CsCl solution they were centrifuged in the gradient at a concentration of 10^{12} phages of each type. After the centrifugation the two bands, separated by more than 0·5 cm (20 drops), were readily visible. For the controls 5 drops of the solution were collected in 1 ml. tris buffer (2-amino-2-hydroxymethyl-propane-1:3-diol) at a distance of about 15 drops from the first (*b*2$^+$ band). Then 5 drops at the middle of each band were collected also in 1 ml. tris buffer. The results of the determination of the DNA content are given in Table 2.

The analysis of the DNA content was made according to the method of Burton (1956). After removing a small portion of the suspension for phage titration, 2 ml. 1 N-perchloric acid and 1 ml. distilled water were added to the tubes containing the drops. Extraction was carried out for 25 min at 70°C, and 2 ml. diphenylamine reagent added per ml. of the extract. The mixture was left at 30°C for 16 hr. Then the absorption of blue light was measured with a Meunier colorimeter with an absorption filter (max. at 590 mμ). A standard calibration curve relating the concentration

† Abortive lysogenization probably explains the turbid appearance of the plaques formed by $\lambda b2c^+$, plaques that cannot be distinguished from those formed by $\lambda b2^+c^+$. In the latter case the turbidity is due to the lysogenic bacteria growing in the center of the plaque. The bacteria in the center of a $\lambda b2c^+$ plaque must have been infected with a large number of phages and thus become abortive lysogens.

of DNA to the light absorption was made for each experiment by means of known dilutions of solution of a known concentration of DNA (Thymonukleinsäure puriss., Fluka, Buchs SG, Switzerland). CsCl in the same concentration as contained in the phage preparations was added to these standard dilutions. Perchloric acid and CsCl react at 20°C and give a precipitate which is soluble at 70°C but precipitates again upon cooling. We have checked that this reaction has no effect upon our measurements.

TABLE 2

DNA content of λb2⁺ and λb2 per plaque former

Exp. no.	Drops	DNA in μg/ml.	Plaque formers $\times 10^{11}$/ml.	g DNA $\times 10^{-16}$ /plaque former
I	$b2^+$ c	33	2·49	1·32
	b2 c	26·3	2·18	1·21
	control	< 1	—	—
II	$b2^+$ c	36·3	2·50	1·45
	b2 c	25	2·22	1·12
	control	< 1	—	—
III	$b2^+$ c	33·2	2·49	1·33
	b2 c	24·2	2·28	1·06
	control	< 1	—	—
mean values	$b2^+$ c	34·2	2·50	1·37
	b2 c	25·2	2·22	1·14

Each value given in the three experiments is the mean of four measurements of DNA and the mean of two independent titrations of the phage.

The DNA content per plaque former was calculated from the results of Table 2. The data show that the $b2^+$ phage contain more DNA per plaque former than the phage *b*2. However, it was necessary to show that $b2^+$ and *b*2 form plaques with the same efficiency before concluding that $\lambda b2^+$ contains more DNA than λ*b*2.

A direct measurement, with the necessary accuracy, of the plating efficiency by means of the electron microscope was not possible because the CsCl had to be dialysed before the preparations could be made and this process might introduce large errors. We determined instead the serum blocking power (SBP) of the highly purified preparations on *b*2 and $b2^+$ by the method of DeMars (1955). Two standard SBP curves were made for *b*2 and two also for $b2^+$ and the plaque formers were measured precisely for several points of the curves. These curves showed that an equal quantity of *b*2 and $b2^+$ plaque formers eliminated an equal quantity of inactivating antibodies. We estimate the accuracy of these determinations to be of the order of 2 to 3%. Thus if we assume that the tails of the *b*2 and $b2^+$ phages have the same antigenic structure, our SBP measurements show that their efficiencies of plating are equal. In other words, to an equal number of plaque formers of *b*2 and $b2^-$ there corresponds an equal number of phage tails and therefore of phage particles, since only complete particles are concentrated at the given density.

Thus the data of Table 2 show that $\lambda b2^+c$ contains more DNA than λ*b*2 *c*. The value of the excess DNA divided by the DNA content of λ*b*2 *c* is

$$\alpha = \frac{2{\cdot}3 \times 10^{-17}\text{g}}{1{\cdot}14 \times 10^{-16}\text{g}} = 0{\cdot}20$$

When this value is used to calculate the change in density by means of (1) one obtains

$$\Delta\rho = \frac{0{\cdot}21 \times 0{\cdot}20}{2 + 0{\cdot}20} = 0{\cdot}019$$

Thus the excess DNA alone is sufficient to explain the increase of the density of $\lambda b2^+$ over than of $\lambda b2$.†

4. Discussion

We have studied the properties of λ phages of an altered density and we have been able to show that the phages of altered density carry a genetic marker $b2$ which determines the density. This genetic marker is situated in the region $m5$ of the genetic map of λ and behaves in crosses in the same way as any other genetic marker. The wild type allele $b2^+$ of the density marker allows the phage to produce stable lysogens while the phages $\lambda b2$ produce abortive lysogens. We have also shown that $\lambda b2^+$ contains about 18% more DNA than $\lambda b2$.

One can speculate about the role played by the extra DNA contained in phages $b2^+$. It cannot contain genes that are essential for the multiplication of the phage since $\lambda b2$ grows as well as $\lambda b2^+$. It seems unlikely that the extra DNA is a duplication for then one would expect, by unequal crossing over of $b2^+$ by $b2^+$, to produce phage of a density larger than that of $b2^+$. No such phage were observed. The only property that the extra DNA confers on the phage $b2^+$ seems to concern lysogenization. Whereas lysogenization with $\lambda b2^+$ results in prophages that multiply as if they were part of the bacterial chromosome, lysogenization with $\lambda b2$ results in prophages that do not multiply as the bacteria divide and are thus diluted out during culture. These $\lambda b2$ prophages however confer on the bacterium which contains them the property of being inducible to lysis after u.v.-irradiation with concomitant production of phages. The extra DNA appears to be fundamentally concerned with the attachment of the prophage to the bacterial chromosome.

One may inquire further as to the function of the marker $b2^+$. It is possible, for instance, that the extra DNA is the marker itself but it is also possible that $b2^+$ only directs the synthesis of the extra DNA which is then attached to the λ chromosome. If it were not attached one could have expected phenotypic mixing, the extra DNA being sometimes wrapped up with $\lambda b2$ DNA into its coat at the maturation of the progeny of a cross $\lambda b^+ \times \lambda b2$. As this is not observed one is led to the conclusion that the extra DNA must be attached to the $\lambda b2^+$ chromosome. The site and manner of this attachment are however completely unknown. The only information which might bear on these points is the presence in a lysate of $\lambda b2^+$ phages of a small proportion (10^{-4}) of phages having the density of $\lambda b2$ which after multiplication have the density associated with $b2^+$. A possible interpretation of this observation would be that $b2^+$ is the site of attachment of the extra DNA but that in rare cases the extra DNA fails to be attached to the phage chromosome.

There may be fundamental, but not as yet understood, connections between the position of $b2^+$ in the region $m5$-h of the genetic map of λ and the stable lysogenization it is able to produce. For it is also in the region $m5$-h that the bacterial *Gal* markers are inserted in the transducing λdg (Arber, 1958), a fact that points to a homology of that region of the λ chromosome and the bacterial chromosome. As the

† An indirect confirmation of these results has been obtained by Arber & Hradecna (private communication). They have been able to show that the X-ray sensitivity of $\lambda b2^+$ is approximately 20% larger than that of $\lambda b2$.

phages $\lambda b2$ never pick up the *Gal* markers of the bacterium, the extra DNA may not only be connected with stable lysogenization but it may constitute the part of the λ chromosome which has homology with the bacterial chromosome.

When u.v. inactivated λ phage infects u.v. irradiated bacteria the phenomenon known as u.v. reactivation (Weigle, 1953) takes place. The reactivated phages contain a large number of mutants. One could ask if these mutants were of altered densities. Preliminary experiments have shown that this is not the case.

Having found a mutation involving the density of a phage particle one may ask if other density mutations do exist. We have indeed found this to be the case (Kellenberger *et al.*, 1960). One of these mutations at least, seems to affect the stability of the phage in certain chemical environments and might be due to an alteration of its protein structure. In crosses with λ wild type phenotypic mixing was observed.

Another density mutation whose locus is in the *c* region of the genetic map of λ affects the immunity of the lysogenized bacteria. When crossed with $\lambda b2$ this new phage gives rise to phages of four different densities, the two new densities appearing through recombination. Thus the recombinant phages can be separated in the density gradient centrifugation from their parents. This offers a technically important method for studying (for instance with ^{32}P) the transfer of parental material to the recombinants. Experiments concerning this are in progress.

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Stabilization to ^{32}P Decay and Onset of DNA Replication of T4 Bacteriophage †

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The genetic loci of T4 bacteriophage become susceptible to mutagenesis by the thymidine analog 5-bromodeoxyuridine a few minutes after the start of intracellular phage growth. This susceptibility presumably signals the onset of phage DNA replication. In order to examine whether the stabilization to decay inactivation which develops at the onset of growth of highly ^{32}P-labeled T4 phages derives from the appearance of replicas of the infecting viral DNA, the time course of susceptibility to mutagenesis and sensitivity to decay inactivation has been followed in one and the same population of T4-infected bacteria. The results of these experiments are:

1. Both the r_{II} and the q loci of T4 become susceptible to 5-bromodeoxyuridine-induced mutagenesis between the 5th and the 6th min after the start of phage growth. Since these two loci are separated by approximately half of the known genetic map of T4, it appears that different parts of the phage genome begin their replication at very nearly the same time.

2. The infective centers develop more than half their ultimate stability to ^{32}P decay inactivation *before* the onset of susceptibility to mutagenesis, i.e. of DNA synthesis. Stabilization to decay inactivation cannot, therefore, result from the appearance of replicas of the infecting phage DNA.

3. If synthesis of protein is blocked by the presence of the antibiotic chloramphenicol from the very outset of phage growth, the development of both susceptibility to mutagenesis and stabilization to ^{32}P decay is prevented. If, however, chloramphenicol is only added 3 min after the start of phage growth, the infective centers can attain an appreciable stabilization to ^{32}P decay while the susceptibility to mutagenesis is still held in abeyance. This demonstrates once more that stabilization precedes DNA replication and cannot, therefore, be the consequence of the appearance of vegetative copies of the infecting phage DNA.

1. Introduction

A *stabilization* process occurs at the initial stages of intracellular growth of ^{32}P-labeled bacteriophages, which progressively reduces the sensitivity of the infective centers to inactivation by decay of the radioactive atoms incorporated into the DNA of the infecting virus (Stent, 1953, 1955). This stabilization can be demonstrated by infecting bacteria with highly radioactive parent phages and then allowing phage development to proceed for various times before freezing and storing samples of the infected culture at − 196°C. In the frozen state, the slow radioactive decay can proceed for days or weeks at different well-defined stages of virus reproduction and the survival of the infective centers in each sample can be followed by thawing and

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assaying portions of the frozen samples from day to day. Such experiments show that the infective centers frozen just after infection are inactivated by radioactive decay at nearly the same rate as the free ^{32}P-labeled parent bacteriophages and that infective centers frozen at subsequent stages are inactivated more and more slowly the later intracellular phage growth is arrested.

The mechanism underlying the gradual ^{32}P-decay stabilization—or that of the formally analogous increase in resistance to inactivation by ultraviolet light (Luria & Latarjet, 1947; Benzer, 1952)—is still not understood, despite numerous attempts to resolve this problem (for reviews, cf. Stent, 1958; Stahl, 1959; Stent & Fuerst, 1960). One reasonable explanation, favored by many since the discovery of this phenomenon, is that the appearance in the infected cell of *copies* of the infecting viral genome produces the great increase in radioresistance of the vegetative phage (Symonds & Ritchie, 1961). Thus, stabilization would in some way be a consequence of phage DNA replication. This explanation became all the more plausible when it was found that during the intracellular growth of bacteriophages T2 and T4 the synthesis of phage-specific DNA begins at just about the same time as the stabilization reaction. In the present work we have examined more closely whether in T4-infected bacteria the evolution of radioresistance does indeed coincide with the onset of phage DNA synthesis. For this purpose, tests for stabilization to ^{32}P decay and for replication of the infecting phage genome were carried out *in one and the same population* of infective centers. The results of these experiments show rather clearly that most of the stabilization reaction takes place *before* the start of phage DNA synthesis and that, therefore, the reduction in radiosensitivity cannot derive from the appearance in the cell of replicas of the infecting genome.

2. Materials and Methods

Phage strains. For the principal experiments, a double mutant strain of coliphage T4(B) was employed which carried the 5-bromodeoxyuridine-induced r_{II} mutation previously described (Pratt & Stent, 1959) and a "low acriflavin resistance", or *ac*, mutation selected on agar containing 0·25 μg/ml. acriflavin (Susman & Edgar, personal communication). For genetic mapping, the following were used: the above T4(B)r_{II} mutant, an unnumbered T4(B)r_{I} mutant, T4(D) mutants r_{67} (Brenner & Barnett, 1959) and h_{41} (Edgar, 1958), and several T4(B) and T4(D) *q* mutants (Susman & Edgar, personal communication) resistant to the high level of 2·75 μg/ml. acriflavin in the agar when in combination with *ac*. All strains used in the crosses carried the *ac* mutation.

Bacterial strains. Highly ^{32}P-labeled T4 stocks were grown on *Escherichia coli* strain B/r (Witkin, 1951); *E. coli* strain R2 (Hershey, Dixon & Chase, 1953) was the host organism for the growth experiments to be described and for the genetic crosses. The term *standard suspension* of R2 bacteria refers to a culture grown to a density of 10^8 cells/ml. in MI medium, centrifuged and washed twice, resuspended in MI medium without glucose or casamino acids at a density of 5×10^8 cells/ml. and starved for 0·5 hr. Phage assays were carried out on agar seeded with either strain S (Hershey & Rotman, 1949), strain K12(λ)-112 (Benzer, 1955), strain K12S/4 (Edgar, 1958), or strain BB (McFall & Stent, 1958) as explained in the experimental procedures.

Temperature. The temperature of incubation was 37°C in all experiments.

Media. MI medium is the glucose-salts medium fortified with vitamin-free casamino acids previously described (Pratt & Stent, 1959). *Casamino acids-glycerol* medium is that devised by Fraser & Jerrel (1953). *Salt-free nutrient broth* contains 8 g Difco nutrient broth and 1 g glucose per l. distilled water.

Growth of radioactive phage. The "nutrient-free" highly ^{32}P-labeled stocks of T4 were grown according to the methods previously described (Stent & Fuerst, 1955; Uchida & Stent, 1960*a*).

Reagents. Chloramphenicol was the crystalline product of Parke, Davis & Co. Thymidine and 5-bromodeoxyuridine were purchased from the California Biochemical Corporation and acriflavin neutral from Mann Research Laboratories, Inc.

3. Experimental

(a) *Experiment I: stabilization and mutagenesis*

It was discovered by Litman & Pardee (1956, 1960) that the thymine analog 5-bromouracil, or its nucleoside 5-bromodeoxyuridine (BD), is a potent mutagen for bacteriophages. Since bromouracil is incorporated extensively into the DNA of bacteriophages issuing from infected bacteria growing in the presence of BD (Dunn & Smith, 1954, 1957) and since the frequency of mutants observed under various physiological conditions generally increases in proportion to the amount of analog incorporated, it seems likely that the mutagenic effect is a consequence of the introduction of BD into the vegetative phage DNA. This inference is also supported by the finding of Litman & Pardee (1960) that the presence of BD in a phage-infected bacterial culture is without mutagenic effect at the earliest stages of phage development, before the time at which synthesis of phage-specific DNA begins in the infected cells (Hershey *et al.*, 1953; Vidaver & Kozloff, 1957). Furthermore, removal of BD from the growth medium of infected bacteria in which rapid phage DNA synthesis is already under way, stops the mutation (Pratt, unpublished observations). Thus the presence of BD and the synthesis of phage DNA must be contemporaneous if mutagenesis is to be induced by the analog. The genetic characteristics of bacteriophage mutants induced by BD have been studied in some detail by Freese (1959), who found that most of such mutants can also be induced to sport *reverse* mutants to their original wild type by growth in the presence of BD.

These facts indicate that it is possible to test whether genetic loci carried by the phage DNA are being replicated at any stage of intracellular phage growth by *transient* exposure of the infected bacteria to BD: if an increased number of mutants is found among the phage progeny ultimately produced, then replication of the viral genome—i.e. synthesis of DNA molecules bearing the genetic information—must have taken place during the interval that the mutagen was present in the culture (Brenner & Smith, 1959; Litman & Pardee, 1959). This method is especially useful for determining, more easily than through direct chemical analysis of phage-infected bacteria, the exact timing of the onset of replication of the viral genome at early stages of the latent period. Furthermore, as Dr. Sydney Brenner suggested to us, it is also possible to determine in this way whether different genetic loci carried in the phage genome actually start their replication at the same moment of phage development and whether, under some special conditions of phage growth to be described later in this communication, a *partial replication* of the viral genome may occur, so that replicas of some genetic loci accumulate in the infected cell while other loci remain singular.

In order to relate the time course of decay stabilization to that of replication of the genetic loci carried in the DNA of the infecting virus, an experiment was carried out in which decay inactivation and BD-induced mutation were followed in one and the same population of infective centers. The genetic loci employed for this purpose were the r_{II} locus (Benzer, 1955), at which the reverse mutation of a BD-induced mutant to the r^+ state was examined, and the q locus, at which the forward mutation

from the wild q^+ to the mutant q state was examined. Phages carrying the r_{II} mutation are unable to grow on strain *E. coli*, K12(λ), on which the r_{II}^+ wild type is able to grow; phages carrying both the q mutation and an *ac* mutation at a genetic locus entirely different from q are able to grow in the presence of 2·75 μg/ml. acriflavin, an acridine dye concentration which completely prevents the reproduction of the q^+

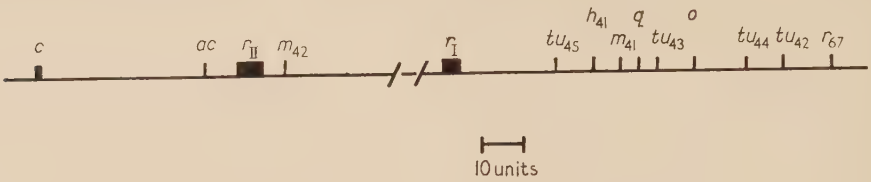

FIG. 1. The genetic linkage map of T4 (Brenner, 1959; Brenner & Barnett, 1959; Streisinger & Bruce, 1960; Susman & Edgar, personal communication).

wild type. Phages carrying only the *ac* mutation possess a low-grade resistance to acriflavin, in that they are able to grow in the presence of dye concentrations of 0·25 μg/ml. (Susman & Edgar, personal communication). Table 1 presents the results of crosses of phages carrying the q mutation against phages carrying other known mutant loci. The phenotype and evident linkage to h_{41} of this q mutant indicate that it is the same as that previously located on the T4 linkage map at the position shown in Fig. 1 (Susman & Edgar, personal communication).

TABLE 1

The proportion of recombinants produced in crosses of the high acriflavin resistance q *locus to other known genetic loci of T4.*

	Number of progeny				
	parental genotypes		recombinant genotypes		
Cross	$q\ x^+$	$q^+\ x$	$q\ x$	$q^+\ x^+$	Recombinant %
$q \times r_I$	402	—	192	—	33
$q \times r_{67}$	50	80	10	34	25
$q \times r_{II}$	43	63	39	20	36
$q \times h_{41}$	47	28	6	5	13

Procedure. A culture of 5×10^8 R2 bacteria/ml. was infected at a multiplicity of 3 phages of each parental genotype per cell. Adsorption was allowed to proceed for 5 min, by which time less than 10% of the phage input remained unadsorbed. These remaining unadsorbed phage were neutralized with anti-T4 serum. The infective centers were then diluted 5×10^5-fold in nutrient broth and incubated for 60 min, before chloroform was added to induce lysis and the progeny phage were assayed. In the cross of q to r_I the proportions of r and r^+ were determined only among the q progeny, by plating the progeny phage on agar supplemented with 2·75 μg/ml. acriflavin and seeded with strain BB. In the crosses of q to r_{67}, r_{II}, and h_{41}, the progeny were assayed on ordinary acriflavin-free agar seeded with strain S, on which the genotype with respect to r may be scored. Plaques from these plates were picked and tested individually for the h genotype on ordinary agar seeded with strain K12S/4, or for the q genotype on acriflavin agar seeded with strain BB.

With similar experimental procedures, control crosses involving other unlinked genetic loci in T4 yielded about 30% recombinants.

Bacteria were infected singly (actual multiplicity = 0·20 phage per bacterium) with a highly ^{32}P-labeled stock of T4$r_{II}ac\ q^+$ phage and BD mutagen was added to the culture at the start of intracellular phage growth. At one minute intervals, samples were withdrawn from the infected culture and a set of aliquots of each sample was frozen and stored, so that the sensitivity to ^{32}P-decay inactivation of

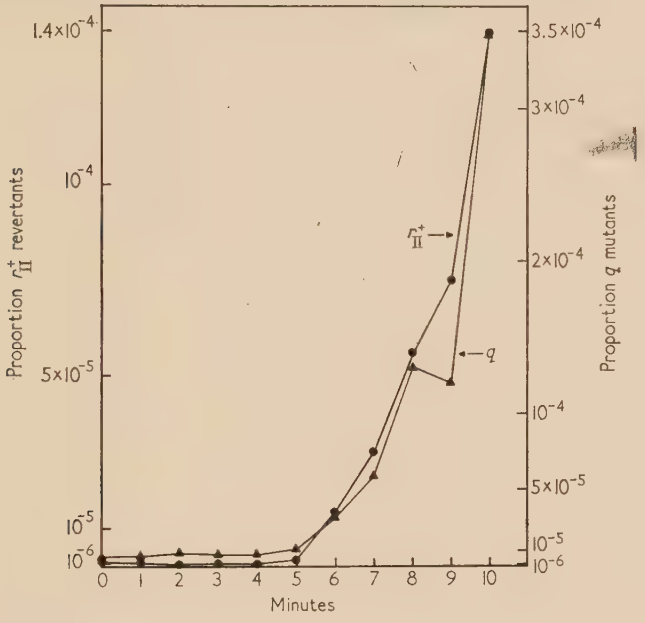

FIG. 2. The relative proportion of r_{II}^+ revertants (left hand ordinate) and q mutants (right hand ordinate) among the phage progeny liberated from T4$r_{II}ac\ q^+$ infected bacteria incubated in the presence of BD from the time of infection until the time indicated on the abscissa.

Procedure. A standard suspension of R2 bacteria was infected with 10^8 viable phages/ml. of a "nutrient-free" radioactive stock of T4(B)$r_{II}ac\ q^+$ labeled at a specific ^{32}P activity of about 1400 mc/mg. After adsorption of the phage had proceeded for 2·5 min the culture was diluted to a cell density of 2×10^8/ml., BD was introduced into the culture to a final concentration of 40 μg/ml. and phage growth was initiated by addition of glucose and vitamin-free casamino acids ($t = 0$). The culture was then incubated under vigorous aeration and samples were diluted 10-fold at 1 min intervals into ice-cold casamino acids-glycerol medium (storage tubes). One portion of each storage tube was diluted twenty-fold into salt-free nutrient broth fortified with 200 μg/ml. thymidine and returned to 37°C for another 60 min, when lysis of each sample was induced by chloroform. The titer of r_{II}^+ revertants in each lysate was determined by assay on tryptone broth agar seeded with strain Kl2 (λ)-112; the titer of q mutants by assay on tryptone broth agar containing 2·75 μg/ml. acriflavin, seeded with strain BB; and the total titer by assay on tryptone broth agar seeded with strain S. Under these plating conditions, the q mutants form a wide spectrum of plaque types; however, most of the q mutations observed here must concern the same general locus on the T4 linkage map, since in 5 crosses involving 6 randomly selected q mutants of independent origin and different plaque morphology on acriflavin agar no q^+ recombinants were found among some 500 progeny examined.

the infective centers could be followed. At the same time, the mutagenic action of BD was terminated in another portion of each sample by dilution of the culture into thymidine-supplemented medium (Litman & Pardee, 1960). This was then incubated further until the appearance of infective progeny, so that the relative frequency of r_{II}^+ and q mutants induced by the early exposure of the culture to BD could be determined.

The results of this experiment are shown in Figs. 2, 3(a) and 3(b). Figure 2 presents the frequencies of the two mutant types found among the phage progeny in samples from which the BD mutagen had been removed at progressively later times. It is apparent that among phages in samples from which BD was removed during the first 5 min of intracellular phage growth there is no increase in the proportion of either r_{II}^+ or q genotypes over the spontaneous background level. The sample which was exposed to BD until the 6th min shows a significantly higher proportion of

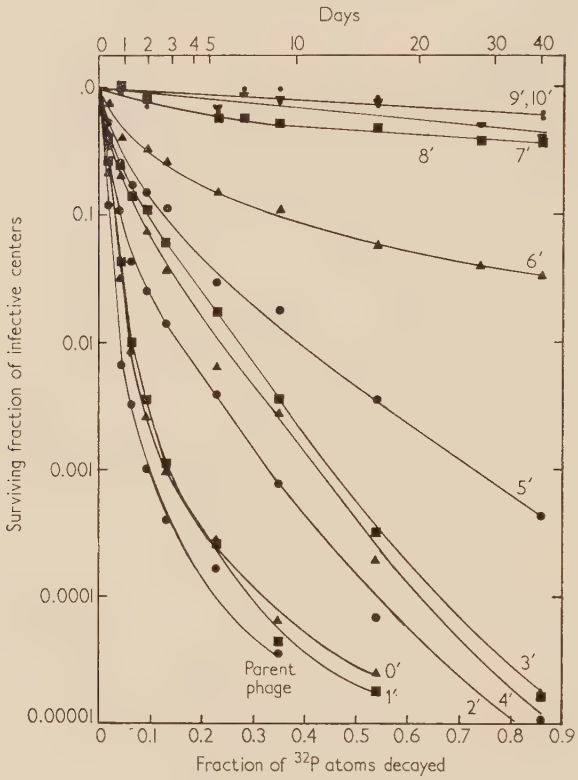

FIG. 3(a). The survival with radioactive decay of infective centers in which intracellular growth of ^{32}P-labeled T4 has proceeded for the number of minutes indicated on each curve.

Procedure. Numerous aliquots of each storage tube described under Fig. 2 as well as portions of the parental ^{32}P-labeled T4 stock diluted in casamino acid-glycerol medium, were frozen and stored at — 196°C. From day to day, one of the frozen samples of each aliquot was thawed and plated at once on tryptone broth agar seeded with strain S for assay of the number of surviving infective centers.

mutants at both loci, and subsequent samples contain an ever-increasing frequency of mutants with later and later removals of BD from the culture. This result shows that replication of the DNA molecules bearing these two genetic loci situated at a considerable distance from each other on the genetic linkage map *begins at the same time*, namely between the 5th and 6th min of intracellular phage development. This estimated time for the onset of phage DNA replication could actually be somewhat in error, if the mutagenic action of BD is not really arrested at the very moment that the infected culture is diluted out of BD into thymidine-supplemented medium. That is, if after removal of BD from and addition of thymidine to the growth medium

bromouracil-containing nucleotides still persist temporarily in the intrabacterial metabolic pools, then some of the induced mutations observed in this experiment might have occurred only after BD had already been removed from the medium. Such delayed mutations would cause our estimate of the onset of DNA synthesis to be *too early* relative to the true time, i.e. replication of the two genetic loci would actually commence at a time later than the 5th to 6th min. It is highly unlikely, on the other hand, that our estimate of the onset of DNA synthesis is *too late*

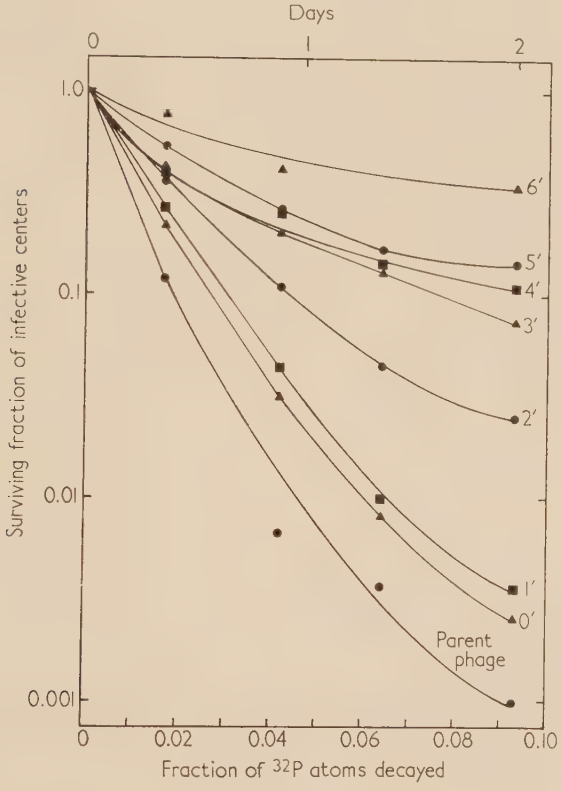

FIG. 3(b). Some of the data of Fig. 3(a) replotted with a greatly expanded abscissa.

relative to the true time, as it would be if BD penetration into the cell and entrance into the appropriate phage DNA precursor pools required so long that mutation could only begin at the 5 to 6 min time even if DNA synthesis began earlier. For in control experiments in which BD was presented to T4-infected cultures at late stages of their latent period, mutant DNA appeared in mature, infective progeny phages within 2 min after addition of the mutagen to the medium (Pratt, unpublished results). Since most of these 2 min are required for the maturation of the vegetive phage DNA into structurally intact infective virus particles (Koch & Hershey, 1959), it seems most likely that BD enters the phage DNA precursor pools as an effective mutagen within a few seconds of its addition to the culture medium.

We may now consider the sensitivity of this method for detecting the onset of phage DNA synthesis. It can be seen in Fig. 2 that in the sample from which BD had

been removed at the 10th min, the proportion of r_{II}^+ revertants had risen from the spontaneous background level of about 10^{-6} revertants per phage to $1{\cdot}4 \times 10^{-4}$ revertants per phage, an approximately 140-fold increase. Since phage DNA accumulates in the infective centers at a constant rate of about 5 phage equivalents per min (Hershey *et al.*, 1953; Hershey & Melechen, 1957), some 25 phage equivalents of new phage DNA have been synthesized between the 5th and 10th min, corresponding to about (log 25/log 2) = 4·6 rounds of geometric replication of the genetic material (Luria, 1951). Inasmuch as each round of replication in the presence of BD should contribute equally to the final proportion of mutants found (Novick & Szilard, 1950), the rate of mutation per round, and in particular during the first round of doubling of the DNA of the infecting phage, can be estimated to about $(1{\cdot}4 \times 10^{-4}/4{\cdot}6) = 3{\cdot}0 \times 10^{-5}$ r_{II}^+ per phage, or an approximately 30-fold increase over the spontaneous background of reverse mutation. Since a threefold increase over background in revertant frequency is easily measurable, this method can signal the moment when an average of as little as 0·1 round of replication of the infecting genome has occurred. On this basis, we infer that in experiment I an average of less than 0·1 round of replication has occurred in the 5 min sample, while in the 6 min sample the frequency of $1{\cdot}4 \times 10^{-5}$ r_{II}^+ revertants per phage shows that by the 6th min of intracellular growth an average of about 0·5 phage equivalents of new DNA has been formed per infected cell.

Figure 3(a) presents the survival with ^{32}P decay of the samples frozen at various stages of intracellular growth and thawed from day to day for assay of the remaining number of infective centers. It can be seen that the usual stabilization phenomenon is again observed here: in samples frozen immediately after infection, the infective centers disappear at a rate similar to the rate of ^{32}P decay inactivation of the free parental T4 phage, whereas the infective centers of the other samples are inactivated more slowly the later the time at which intracellular phage development has been arrested. Stabilization has progressed so far by the 7th and 8th min of phage growth that the infective centers frozen at this time are inactivated at only 1% the rate of death of the radioactive parent T4, but it should be noted that even in these samples at least half of the infective centers are still subject to inactivation, albeit at a very slow rate. Some inactivation can still be perceived at the 9th and 10th min of phage growth, so that even at this late time stabilization does not appear to be complete.

In order to present more clearly the initial time course of stabilization in this experiment, some of the data of Fig. 3(a) have been replotted in Fig. 3(b) with a greatly expanded abscissa. Evidently the samples frozen immediately after infection are not inactivated at exactly the same rate as the free parental phage but at only 0·7 that rate. A further stabilization begins at the 2nd min of phage growth and by the 5th and 6th min, the bulk of the infective centers is inactivated at only 0·3 and 0·2 of the rate of death of the nought minute infective centers. That is to say, a very considerable stabilization has already occurred *before* any appreciable replication of the genetic material of the radioactive parental virus can have taken place. If, as we indicated as possible above, our estimate of the onset of DNA synthesis is somewhat too early, then an even greater degree of stabilization precedes the beginning of replication. In any case, by the 7th min, when, on the basis of the BD-induced r^+_{11} reversion frequency one estimates that the first round of replication has just been completed, only 1% of the initial radiosensitivity remains.

The survival curves in Fig. 3 are not simple exponentials, but are concave curves which exhibit a decrease in slope at later stages of decay. The concavity of the survival curve of the free T4 phage must reflect a heterogeneity in labeling among the individuals of the parent stock. This heterogeneity probably derives from the fact that for preparation of T4 particles containing the very high specific ^{32}P activities employed here it is not feasible to grow up the radioactive bacterial host culture from a small inoculum in the highly labeled medium. At the time of their infection, therefore, the bacteria contain appreciable amounts of non-labeled phosphorus, which they then donate to the phage progeny (Cohen, 1948; Stent & Maaløe, 1953); it is the non-uniform distribution of this bacterial contribution which is most likely responsible for the heterogeneity in ^{32}P-decay sensitivity observed. The concavity of the infective center survival curves at later stages of phage development could reflect a non-synchronous progress of the stabilization reaction in different fractions of the population of infected cells, but it could also be an epiphenomenon of the heterogeneous labeling of the parent phage.

In connection with experiment I we have examined the effect of ^{32}P decay on the appearance of BD-induced r_{II}^+ revertants in the sample frozen at the 6th min of phage growth, at a time, when a partial stabilization had occurred and when the first increase over the spontaneous revertant background became detectable. The purpose of this study was to see whether these first induced mutants are liberated from a special fraction of the infective centers, in which phage growth, and hence the stabilization reaction, had progressed more quickly than in the majority of the population. If this were the case, then the *proportion* of r_{II}^+ revertants among the phages liberated from the stored samples at various stages of radioactive decay should display a continuous *rise*, as the majority of decay-sensitive infective centers which do not yet contribute to the mutant yield, are inactivated more rapidly than the advanced minority of decay-resistant infective centers which produce the mutants. Accordingly, from day to day at various stages of ^{32}P decay, portions of the sample frozen at the 6th min of growth were thawed and assayed for the surviving infective centers and the infected bacteria were then diluted into thymidine-supplemented nutrient broth and incubated for 60 min before being lysed with chloroform. The number of r_{II}^+ revertants and the total phage yield liberated from the surviving infective centers was assayed. The results of these determinations are presented in Table 2.

First of all, it is interesting to note that the total burst size (i.e. the number of progeny produced per surviving infective center) remains relatively constant as radioactive decay proceeds. This indicates that ^{32}P disintegrations in the viral DNA, which are lethal if they occur at the time of infection but which are no longer lethal if they occur at the 6th min of growth, do not produce any very serious *sub-lethal* effects once the stabilization reaction has set in. Secondly, and more important, it is clear from these results that although a fivefold increase in the proportion of revertants was found at early stages of ^{32}P decay and at late stages only single mutant plaques could be scored, finally, when only 3% of the infective centers survived, no noticeable increase in the proportion of revertants among the progeny of surviving infective centers had occurred. The explanation of the rather wide day-to-day variations in the actual proportion of r_{II}^+ revertants observed is probably that since induced mutation just begins at the 6th min, so few mutational events occur in each of the final lysates that large fluctuations are produced in the number of mutants

per sample. In any case, there is no doubt that ^{32}P decay reduces the total number of r_{II}^+ revertants in the culture, and hence the induced mutants cannot be the issue of infective centers in which stabilization was complete at the 6th min. It is tempting to draw the inverse conclusion that stabilization is not yet complete in those infective centers in which the first phage DNA replica has already been made. This is, however, uncertain, because of the already mentioned possibility of a time lag between the removal of BD and the cessation of mutagenesis, which could mean that even those cells of the 6 min sample which produced revertants did not actually do so until after that time.

TABLE 2

Proportion of r_{II}^- revertant phage in lysates of infective centers diluted away from 5-bromodeoxyuridine and frozen at the 6th minute of phage growth, stored at — 196°C while ^{32}P decay proceeded, then thawed and allowed to produce progeny phage.

Days of storage	Fraction of ^{32}P atoms decayed	Surviving fraction of infective centers	Burst size per surviving infective center	Actual number of revertant plaques counted	Proportion r_{II}^+ revertants in lysate
0	0	1·0	34	120	$1{\cdot}2\times10^{-5}$
0·9	0·04	0·41	35	95	$2{\cdot}3\times10^{-5}$
2·0	0·09	0·33	39	64	$1{\cdot}7\times10^{-5}$
5·4	0·23	0·15	24	31	$6{\cdot}2\times10^{-5}$
9·0	0·35	0·11	13	27	$6{\cdot}4\times10^{-5}$
16	0·54	0·06	21	1	$\sim 3\times10^{-6}$
28	0·74	0·04	25	1	$\sim 3\times10^{-6}$
41	0·86	0·03	18	1	$\sim 6\times10^{-6}$

Nevertheless, these results from the 6 min sample reinforce the general conclusion of experiment I that the stabilization reaction and the appearance of replicas of the phage DNA are not the same thing. If, on the one hand, DNA synthesis *has* begun in some of the cells by 6 min then ^{32}P decay in the parental polynucleotide chains can still destroy an infective center already containing a DNA replica; if, on the other hand, DNA synthesis *has not* yet begun in any of the cells by the 6th min, then more than 80% of the stabilization reaction precedes the appearance of any DNA replicas.

(b) *Experiment II: stabilization and the "early" proteins*

At the outset of intracellular growth, an "early" protein is synthesized in T2 and T4 infected bacteria whose presence is necessary before synthesis of phage DNA can begin. This had been demonstrated by adding the antibiotic chloramphenicol, which arrests protein synthesis (Wisseman, Smadel, Hahn & Hopps, 1954), to the infected culture at various times after the start of phage growth and then assaying for the amount of phage-specific (i.e. hydroxymethylcytidylated) DNA formed upon further incubation. If chloramphenicol is added before the 4th min of phage growth, no phage DNA appears in the culture. If chloramphenicol is added only after that time, phage DNA synthesis does proceed, the ultimate rate of synthesis being greater, the later the antibiotic is added. No infective progeny particles appear, of course, until the antibiotic is again removed and synthesis of the precursor protein of the progeny virus particles is allowed to occur (Burton, 1955; Tomizawa & Sunakawa,

1956; Hershey & Melechen, 1957). The phage DNA synthesized in the presence of chloramphenicol accumulates in a pool of precursors and is withdrawn from that pool for maturation into infective progeny after removal of the antibiotic (Hershey & Melechen, 1957; Tomizawa, 1958). Exposure of T2-infected bacteria to BD only during the time of the accumulation of phage DNA in the presence of chloramphenicol causes the appearance of a high proportion of mutants among the phage progeny formed once protein synthesis has been allowed to resume, showing that under these conditions, BD is an effective mutagen and the DNA synthesized is genetically "active" (Brenner & Smith, 1959; Litman & Pardee, 1959). No doubt, this "early" protein represents, at least in part, the complex of metabolic enzymes whose *de novo* formation is now known to be induced by the infecting virus and which is understandably required for synthesis of phage-specific DNA (Flaks & Cohen, 1957; Kornberg, Zimmerman, Kornberg & Josse, 1959). The "early" protein also plays an important role in the stabilization process of the vegetative phage, since very little reduction in decay sensitivity takes place upon incubation of bacteria infected with ^{32}P-labeled T2 in the presence of chloramphenicol. After the antibiotic is removed and protein synthesis is allowed to resume, the infective centers stabilize to decay inactivation in the normal way. However, synthesis of the early protein is not only required for the *start* of the stabilization process but *throughout its course*, since chloramphenicol added to the culture a few minutes after the onset of intracellular phage growth arrests the infective centers at a stage intermediate between full sensitivity and complete resistance to decay inactivation (Uchida & Stent, 1960*a*). Further study of the relation of protein synthesis to the stabilization process showed that the "early" protein contains an individually specific component which every phage genome must be allowed to form for itself before it can attain the decay-resistant state (Uchida & Stent, 1960*b*). Thus the role of the early protein in the stabilization reaction cannot readily be explained exclusively in terms of the *de novo* synthesis of enzymes for phage-specific DNA metabolism.

One of the notions which could account for these findings is that a component of the "early" protein is part of a structure which "grows" along the DNA of the infecting phage and in this way both "activates" the viral genome for its replication and stabilizes it against destruction by disintegration of incorporated ^{32}P atoms. From this point of view, an infective center in which phage development has reduced the decay sensitivity to some fraction x of its initial value would correspond to a cell in which growth of this protein has already proceeded along a fraction $(1\text{-}x)$ of the infecting phage DNA complement and in which $(1\text{-}x)$ of the phage genome is thus ready to replicate. Stoppage of protein synthesis in the culture at this point would prevent any further stabilization and allow replication of only the already activated part of the genome upon further incubation. The experiment to be presented now has been carried out to examine this possibility.

As in experiment I bacteria were infected singly (actual multiplicity = 0·25 phage per bacterium) with highly ^{32}P-labeled T4$r_{II}ac\,q^{+}$ phage. The culture was then incubated in nutrient medium and chloramphenicol was added to different samples of the culture at 1 min intervals during the first 10 min of intracellular phage development. After 10·5 min of phage growth, BD mutagen was added to each of these samples and incubation was continued until the 40th min, when aliquots of each sample were frozen and stored so that the sensitivity to ^{32}P decay of the infective centers could be followed. At the same time, both BD and chloramphenicol were

removed from another portion of each sample by dilution into thymidine-supplemented broth and these were then incubated until appearance of infective progeny, so that the relative frequency of r_{II}^+ and q mutants induced by exposure of the culture to BD in the presence of chloramphenicol could be determined.

The results of this experiment are shown in Figs. 4 and 5. Figure 4 presents the BD-induced frequencies of r_{II}^+ and q mutant types found among the phage progeny

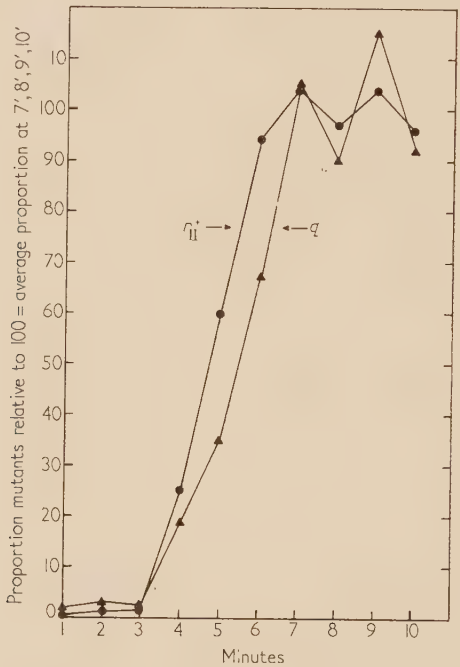

FIG. 4. The relative proportion of r_{II}^+ revertants and q mutants, expressed as percentage of the maximum frequency, among the phage progeny liberated from T4$r_{II}ac\ q^+$ infected bacteria to which chloramphenicol is added at the time indicated on the abscissa and which are exposed to BD mutagen only in the presence of the antibiotic.

Procedure. A standard suspension of R2 bacteria was infected with $1{\cdot}2 \times 10^8$ viable phage/ml. of a "nutrient-free" stock of T4(B)$r_{II}ac\ q^+$ labeled at a specific ^{32}P activity of about 1400 mc/mg. After adsorption of the phage had proceeded for 2·5 min, the culture was diluted to a cell density of 2×10^8/ml. and phage growth was initiated by addition of glucose and vitamin-free casamino acids ($t = 0$) and the culture was incubated under vigorous aeration. At intervals of 1 min, 200 μg/ml. chloramphenicol was added to different samples of the culture, followed by addition to all samples of 200 μg/ml. BD at $t = 10{\cdot}5$ min. The samples were then incubated further until $t = 40$ min, when exposure of the infected bacteria to both mutagen and antibiotic was terminated by two successive centrifugations of the cells and resuspension in ice-cold casamino acids-glycerol medium (storage tubes). As in experiment I, one portion of each storage tube was diluted tenfold into thymidine-fortified broth, incubated further, and the relative proportions of r_{II}^+ revertants and q mutants in the lysate of each sample were determined.

in samples of the infected culture to which chloramphenicol had been added at different times and which had all been exposed to BD from the 10th to the 40th min before chloramphenicol was once more removed. The observed mutant frequencies are shown here as percentages of the *maximum* mutant frequency found upon late addition of chloramphenicol to the culture (taking as 100% the average of the mutant frequency found in the last 4 samples). This normalization of mutant frequencies allows not only a direct graphical comparison of relative mutagenesis at

the two different loci but affords also a quantitative appreciation of the degree to which synthesis of "early" protein, before addition of the antibiotic, has already prepared the infective centers for later phage DNA synthesis. It is evident from Fig. 4 that the two loci r_{II} and q^+ behave in very much the same way: no induced mutation is discernible at either locus if chloramphenicol is added to the culture at the 1st, 2nd or 3rd min of phage development. Addition of chloramphenicol at progressively

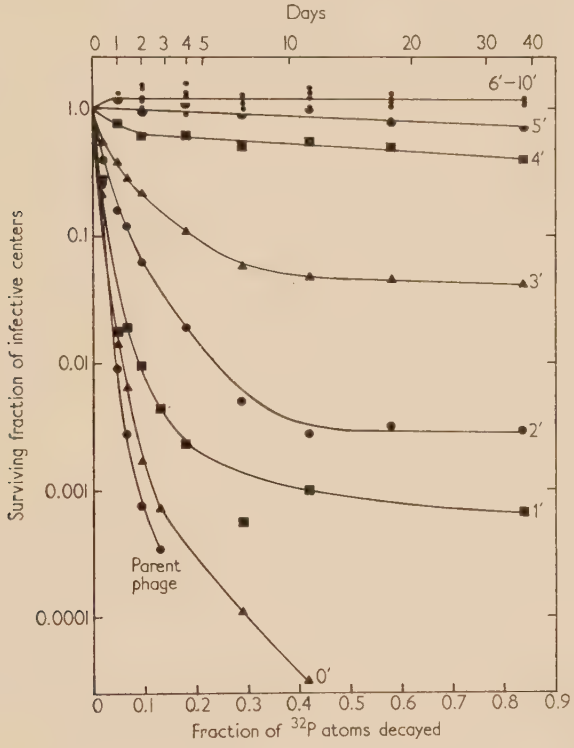

FIG. 5. The survival with radioactive decay of infective centers to which chloramphenicol is added after intracellular growth of ^{32}P-labeled T4 has progressed for the number of minutes indicated on each curve and which are frozen 40 min after infection.

Procedure. As in Experiment I, numerous portions of each storage tube described under Fig. 4 were frozen, stored at — 196°C, and the number of surviving infective centers was assayed from day to day.

later times then allows the appearance of more and more BD-induced mutants, each minute of delay in chloramphenicol addition promoting about 25% of the final mutational effect, until at the 7th min the final mutant level for each locus has been reached. The following conclusions can be drawn from these results:

1. A 30 min exposure of the infected bacteria to BD while phage DNA synthesis has been prevented by early addition of chloramphenicol leaves no "memory" of the mutagen in the cells. Hence removal of BD from the cells at the time of resumption of protein synthesis is evidently very efficient.

2. A 30 min exposure of the infected bacteria to BD after addition of chloramphenicol at a time when only part of the "early" protein has as yet been made and DNA synthesis can proceed at only a fraction of its maximum rate engenders the same

small percentage of the maximum possible mutagenesis at both r_{II} and q^+ loci. This is contrary to what is to be expected if it is postulated that during the 4 min of intracellular growth required for preparing the infected cell for maximum phage DNA synthesis the infecting phage DNA is activated by the orderly growth of a protein from one end of the linkage map to the other and if already activated parts of the genome can replicate in the absence of further protein synthesis. Since r_{II} and q^+ are situated toward opposite ends of the known T4 linkage map, one of the two loci should, under this hypothesis, become activated before the other. Hence it may be concluded that either there is no partial activation of the infecting DNA by gradual growth of a component of the "early" protein, or if such "activation" exists, then it does not proceed in an orderly manner from one end of the phage genome to the other in every infected cell.

Figure 5 presents the survival with ^{32}P decay of the samples of the infected culture to which chloramphenicol was added at different times and which, after further incubation until the 40th min, were frozen and thawed from day to day for assay of the remaining infective centers. It can be seen in this figure that the previous finding that protein synthesis is necessary throughout the stabilization process has been confirmed: infective centers to which chloramphenicol was added before the 5th min of intracellular phage growth are arrested at intermediate stages between the full ^{32}P decay sensitivity of the parental virus and complete stabilization. Let us now focus our attention on the sample to which chloramphenicol was added at the 3rd min of phage growth. Its ^{32}P decay survival curve (whose complex shape we shall discuss momentarily) indicates that at least 80% of the infective centers are inactivated at less than 25% the rate of death of the nought-minute infective centers, i.e. that here stabilization is 75% complete. From the point of view of activation and stabilization by growth of a protein along the infecting DNA, this would mean that 75% of the viral genome is ready to replicate without further protein synthesis. However, the relative mutant frequencies of Fig. 4 show that, in fact, in this sample no replication of either one of the two distant genetic loci proceeds in the presence of the antibiotic. Thus it follows that the hypothesis proposed at the outset of this experiment cannot be true: the fractional stabilization of the infective centers observed at various stages of intracellular development cannot represent the growth of a protein along part of the infecting DNA which activates the viral genome and allows it to replicate in the absence of further protein synthesis.

The family of decay survival curves of Fig. 5 (experiment II) differs from that of Fig. 3(*a*) (experiment I) in two important ways. First, if two survival curves bearing the same time parameter are compared, it is evident that the stabilization reaction has not progressed as far in the infective centers directly frozen at a given moment in phage development as in the infective centers to which chloramphenicol was added at that time and which were frozen only at the 40th min after infection. During the critical time while the sensitivity to decay of the infective centers undergoes its most dramatic change, the stabilization manifest in Fig. 5 seems to be about 2 min ahead of that of Fig. 3(*a*), i.e. the period 2 to 5 min in Fig. 5 more or less corresponds to the period 4 to 7 min of Fig. 3(*a*). Secondly, the survival curves of Fig. 5 resemble simple exponentials even less than those of Fig. 3(*a*). With the exception of the survival of the free phage and of the nought-minute infective centers in which no phage development occurred, all the curves of Fig. 5 finally approach an assymptote of zero slope. We believe that both of these differences are produced by the

same cause, namely that chloramphenicol does not act instantly in inhibiting stabilization of ^{32}P decay in the phage-infected bacteria but requires an average time of about 2 min. This time lag, furthermore, is not uniformly distributed over the population, so that after addition of the antibiotic, stabilization is arrested more quickly in some infective centers than in others. For instance, the shape of the 1 min survival curve of Fig. 5 can be explained by envisaging that in 10% of the infective centers the antibiotic requires as much as 4 min to establish its effect and that in 0·1% of the population chloramphenicol acts only after a delay of 6 min. An average delay of 2 min for the action of chloramphenicol also explains the difference in the time of onset of mutagenesis, i.e. DNA synthesis, observed in experiments I and II; for Fig. 2 showed that mutagenesis begins after the 5th min of phage growth, whereas Fig. 4 shows that delaying the addition of antibiotic until after the 3rd min already allows mutagenesis during subsequent incubation.

4. Discussion

The observations reported here eliminate one of the three alternative hypotheses originally offered to explain the progressive reduction in ^{32}P decay sensitivity of the infective centers (Stent, 1955), namely that of *multiplicity reactivation*. Inasmuch as experiments I and II agree in showing that most of the stabilization effect occurs before replication of the infecting DNA begins, it is not possible that the gradual decrease in the lethal fraction of ^{32}P disintegrations reflects a process akin to multiplicity reactivation (Luria, 1947) which protects the genetic information of already duplicated portions of the infecting phage genome against radiochemical destruction. Since previous findings on the role of the "early" protein in the stabilization reaction had already made the multiplicity reactivation hypothesis very unlikely, the alternative was more favorably considered that the gradual decrease in ^{32}P decay sensitivity reflects not the actual replication of the infecting DNA but, instead, its progressive *activation* for replication by a component of the early protein (Uchida & Stent, 1960*a*). The result of experiment II now shows that this hypothesis is also not very satisfactory, because infected bacteria in which stabilization has already progressed very far are not ready to replicate any part of their genome without further protein synthesis.

It would thus appear that two principal alternatives remain to explain the stabilization process. One of these, the lone survivor of the three originally proposed (Stent, 1955), is that at the outset of intracellular growth, the infecting DNA undergoes some progressive change in structure or configuration, which renders it intrinsically much less sensitive to destruction by ^{32}P disintegrations ; continuous protein synthesis would have to be required to achieve this change. The other alternative (Benzer, 1952) is that the stabilization reaction reflects the progressive *phenotypic expression* of the genetic information carried into the cell by the phage genome; so that once enough of the gene product of a certain region of the infecting DNA has accumulated in the host cell cytoplasm, further phenotypic function of that region can be destroyed by lethal ^{32}P disintegrations within it without suppressing the ultimate appearance of infective progeny. The consequences which this hypothesis would entail have already been discussed elsewhere (Uchida & Stent, 1960*a*). Perhaps the last two hypotheses can be fused into one: in the act of giving phenotypic expression, the infecting DNA might experience a structural change which renders it resistant to destruction by decay of its incorporated radioisotope. Unfortunately, the present

experiments do not permit us to distinguish between the remaining alternatives and only leave us with the distinct impression that synthesis of phage DNA has little or nothing to do with the ^{32}P decay stabilization reaction.

We are greatly indebted to Dr. Sydney Brenner for suggesting to us the test for partial replication of the viral genome by induced mutagenesis and also wish to thank him and Dr. Robert S. Edgar for advice on the use of the acriflavin-resistance character, and Dr. Hisao Uchida for help with preliminary experiments.

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Genetic Control of Repression of Alkaline Phosphatase in *E. Coli*

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Synthesis of the enzyme alkaline phosphatase by the standard strain of *E. coli* K12 is repressed when growth occurs in a medium containing excess orthophosphate. Repression of this enzyme is under the control of two regulator genes; one is closely linked to the structural gene, and the other is located in a distant region. The presence of a constitutive mutation in either of the regulator genes prevents repression of the enzyme. The results of *cis-trans* tests show that the constitutive alleles of the two genes are recessive in both *cis* and *trans* positions relative to the structural gene. This suggests that each regulator gene plays an essential role in the formation of a repressor for alkaline phosphatase.

1. Introduction

It has been shown for several bacterial enzymes that the rate of enzyme synthesis is regulated by a mechanism of repression (cf. reviews by Monod, 1959; Pardee, 1961; Vogel, 1961). The most detailed information on repression has been obtained for the enzyme β-galactosidase in *E. coli*. The repression of this enzyme is controlled by two genes, a "regulator gene", *i*, and an "operator gene", *o*. Constitutive mutations can occur in the *i* and the *o* genes. The two classes of constitutive mutations can be distinguished by their behaviour in heterozygotes. In a cell which is heterozygous for the *i* gene, the constitutive allele of the *i* gene is recessive when it occupies either a *cis* or *trans* position relative to the active allele of the structural gene for the enzyme, whereas in a cell which is heterozygous for the *o* gene, the constitutive allele of the *o* gene is dominant in the *cis* position and recessive in the *trans* position. The interpretation of these observations is that the *i* gene specifies the formation of an intracellular repressor, and the *o* gene designates the site at which the repressor acts (Pardee, Jacob & Monod, 1959; Jacob & Monod, 1959; Jacob, Perrin, Sanchez & Monod, 1960).

In the present communication, we report on some properties of the regulating system for the enzyme alkaline phosphatase of *E. coli* (Garen & Levinthal, 1960; Levinthal, 1959; Garen, 1960). It has been shown that the synthesis of this enzyme is repressed in the standard strain when growth occurs in the presence of excess amounts of inorganic phosphate (Torriani, 1960; Horiuchi, Horiuchi & Mizuno, 1959). A procedure for the isolation of constitutive mutants for alkaline phosphatase has been described recently (Torriani & Rothman, 1961). We have carried out genetic mapping of a number of independently isolated constitutive mutations, and have studied the effect of the mutations on the synthesis of alkaline phosphatase in haploid cells and in partial diploid cells heterozygous for constitutive markers.

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2. Materials and Methods

(a) *Nomenclature*

Hfr. male (genetic donor) type (Wollman, Jacob & Hayes, 1956).

F^-. female (genetic recipient) type.

F'. partial diploid male which can transfer its extrachromosomal segment to an F^- partner, producing partial diploid progeny (Jacob & Adelberg, 1959).

S^s and S^r. genetic markers for sensitivity or resistance to streptomycin.

T^+ and T^-. genetic markers for ability or inability to grow without threonine.

L^+ and L^-. genetic markers for ability or inability to grow without leucine.

P^+ and P^-. genetic markers for ability or inability to synthesize alkaline phosphatase.

(b) *Strains*

(The genetic markers listed are those pertinent to the experiments.)

K10. Hfr $S^sT^+L^+$, a derivative of the Cavalli strain which transfers its genome in the direction of *Lac* to *TL*.

Hfr 1. Hfr $S^sT^+L^+$, a strain isolated by Dr. P. Reeves which transfers its genome in the direction of *TL* to *Lac*; requires methionine for growth.

W1. $F^-S^rT^-L^-$, the standard F^- from which all F^- strains used in this study were derived; requires thiamine for growth.

$F'13$. $F'S^s$, an F' strain isolated by Dr. Y. Hirota; requires methionine for growth. The region from *T*6 to *Lac*, which includes the *P* gene, is carried on the extrachromosomal segment.

(c) *Media*

TG medium. $1{\cdot}2\times10^{-1}$ M-tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol); 0·2% glucose; 8×10^{-2} M-NaCl; 2×10^{-2} M-KCl; 2×10^{-2} M-NH_4Cl; 3×10^{-3} M-Na_2SO_4; 1×10^{-3} M-$MgCl_2$; 2×10^{-4} M-$CaCl_2$; 2×10^{-6} M-$FeCl_3$.

TGS medium. TG with 0·1 mg/ml. streptomycin.

TGSE medium. TGS with excess phosphate, $6{\cdot}4\times10^{-4}$ M-KH_2PO_4.

TGSL medium. TGS with limiting phosphate, $6{\cdot}4\times10^{-5}$ M-KH_2PO_4.

TGpS medium. Same as TGS except that β-glycerol phosphate is used as the carbon source in place of glucose; contains excess phosphate ($6{\cdot}4\times10^{-4}$ M-KH_2PO_4).

Penassay medium. Difco prepared formula; contains excess phosphate.

Agar. 1·2% Difco Noble agar.

P medium. 1×10^{-1} M-KH_2PO_4; $1{\cdot}5\times10^{-2}$ M-$(NH_4)_2SO_4$; 8×10^{-4} M-$MgSO_4$; 2×10^{-6} M-$FeSO_4$; 9×10^{-3} M glucose; 100 mg/l. aspartic acid; 1 mg/l. thiamine; KOH to adjust pH to 6·3 (*cf.* Fisher, 1957).

S. medium. 1×10^{-1} M-tris buffer, pH 7·5; $1{\cdot}5\times10^{-2}$ M-$(NH_4)_2SO_4$; 8×10^{-4} M-$MgSO_4$; 2×10^{-6} M-$FeSO_4$.

(d) *Isolation of constitutive mutants*

The isolation procedure has been described elsewhere (Torriani & Rothman, 1961). The agar medium used to select mutants is TG medium with $1{\cdot}1\times10^{-2}$ M-β-glycerolphosphate in place of glucose, and contains $6{\cdot}6\times10^{-4}$ M-KH_2PO_4.

(e) *Genetic crosses for the measurement of recombination frequency*

Overnight cultures in Penassay were subcultured into fresh Penassay and aerated until the density of the Hfr strain was 1×10^8/ml. and of the F^- strain 2×10^8/ml. The F^- culture was centrifuged and resuspended in one-fifth the initial volume of Penassay. Equal volumes of the Hfr and F^- cultures were mixed together and aerated at 37°C for 90 min, then the culture was diluted twenty-fold into Penassay containing 0·1 mg/ml. of streptomycin and aerated until a density of 8×10^8/ml. was reached. The culture was centrifuged, resuspended in tris buffer and plated for recombinants on TGSE agar.

(f) *Scoring of the* R *phenotype of recombinant colonies* (*NPP test*)

The TGSE agar plates were incubated at 37°C for 2 days and then scored by spraying with a solution of 15 mg/ml. *p*-nitrophenylphosphate (NPP) in 1·0 M-tris buffer, pH 8·0. Constitutive (R^-) colonies turn yellow within a few seconds after being sprayed, while repressible (R^+) colonies do not.

(g) *Assay for alkaline phosphatase activity*

For the experiments reported in Tables 4 and 5, 0·5 ml. samples of the toluene-treated bacterial suspensions were mixed with 2·0 ml. of a solution of 1 M-tris buffer, pH 8·0 containing 5 mg NPP/ml. and the rate of change of optical density at 410 mμ at 35°C was measured. The unit of specific enzymatic activity is defined as the change in optical density per minute per unit of cell density (the cell density unit is an optical density of 1·0 at 540 mμ for the original culture before the addition of toluene).

For the experiments reported in Fig. 3, 4, 5 and 6, the procedure was the same as described above, except that the assays were run at 26°C, and the unit of enzymatic activity was based on the direct reading of the sample without correction for the cell density.

(h) *Assay for β-galactosidase activity in cultures*

To a 0·5 ml. sample of a toluenized bacterial suspension was added 2·5 ml. of a solution of 0·8 mg/ml. of *ortho*nitrophenylgalactoside in 0·05 M-sodium phosphate buffer, pH 7·0. The rate of change of optical density at 420 mμ at 37°C was measured. The unit of specific enzymatic activity is the change in optical density per minute per unit of cell density.

3. Results

(a) *Genetic mapping of constitutive mutations*

The positions of a number of independently-occurring constitutive mutations have been located on the genetic map of *E. coli* K12 by means of crosses with Hfr and F⁻ parents. The map positions of the mutations were determined by two procedures, one involving the measurement of recombination frequencies and the other the timing of the passage of the mutations from the donor to the recipient parent (Wollman *et al.*, 1956).

For the first series of crosses, the Hfr parents were independently isolated R^- mutants of strain K10, and the F^- parent was an R^+ strain. The markers used for selection were T^+L^+ from the Hfr and S^r from the F⁻. (It should be noted that the *S* gene is not transferred with significant frequency by K10.) The proportion of R^- recombinants was measured among the selected $T^+L^+S^r$ progeny. Results obtained with sixteen constitutive mutants are shown in Table 1. It is possible to arrange the mutants in two groups, an *R*1 group that produces about 70% R^- recombinants, and an *R*2 group that produces about 2% R^- recombinants. The high frequency of $R1^-$ recombinants indicates that the mutations in the first group are transferred earlier than, or shortly after, the T^+ and L^+ genes by K10, while the low frequency of $R2^-$ recombinants indicates the mutations in the second group are transferred later than the T^+ and L^+ genes. Thus, there appear to exist at least two distinct regions in which the constitutive mutations occur.

For the second series of crosses, each of the twelve constitutive mutants in the *R*1 group was crossed against an F⁻ strain which contained the *R*1 mutation of mutant C2. The results are shown in Table 2. Eight of the twelve crosses yielded a small proportion of R^+ colonies. These colonies could have derived either from genetic recombinants or from revertants which were present in the parental cultures. As a test of the latter

TABLE 1

Crosses of R⁻ *mutants from Hfr strain K10 against an* R⁺ *strain of F⁻*

Hfr $R^-P^+T^+L^+S^s \times$ F⁻ $R^+P^+T^-L^-S^r$

Hfr parent	Number of $T^+L^+S^r$ recombinants tested	R^- recombinants	Percent R^- among $T^+L^+S^r$
C2	416	252	61
C3	398	278	70
C4	1060	19	1·8
C5	392	260	67
C7	620	475	77
C8	253	177	70
C9	528	390	74
C25	372	6	1·6
C26	277	207	75
C27	289	203	70
C28	221	160	72
C30	319	214	67
C31	527	17	3·2
C32	259	177	68
C33	295	220	74

The mated cultures were plated on TGSE agar (containing excess phosphate). The resulting $T^+L^+S^r$ recombinant colonies were tested with NPP and those which turned yellow scored as R^-.

TABLE 2

Crosses of R⁻ *mutants from Hfr strain K10 against an F⁻ strain containing the* R1⁻ *mutation from C2*

Hfr $R^-P^+T^+L^+S^s \times$ F⁻ $R1^-P^+T^-L^-S^r$ (strain C2 F9)

Hfr parent	R class	Number of $T^+L^+S^r$ recombinants tested	R^+ recombinants	Percent R^+ among $T^+L^+S^r$
C2	$R1$	50,000	0	< 0·002
C3	$R1$	15,500	2	0·013
C5	$R1$	6,600	1	0·015
C7	$R1$	33,480	3	0·010
C8	$R1$	59,780	7	0·012
C9	$R1$	9,000	1	0·011
C26	$R1$	4,895	0	< 0·02
C27	$R1$	18,270	5	0·027
C28	$R1$	14,162	0	< 0·007
C30	$R1$	13,680	1	0·007
C32	$R1$	18,722	0	< 0·005
C33	$R1$	16,285	3	0·019
C4	$R2$	1,466	1022	68
C25	$R2$	585	352	60
C31	$R2$	595	402	68

The mated cultures were plated on TGSE agar, and the resulting colonies were tested with NPP. The colonies scoring as white were picked and replated on TGSL agar, and then tested with NPP. All of the colonies tested scored as yellow, which proves that they were P^+ rather than P^-, and therefore also R^+.

possibility, unmated cultures of five of the Hfr parents (C7, C8, C9, C27, and C33) and the F⁻ parent were screened for R^+ revertants. None was found among a total of more than 15,000 colonies examined from each culture. As an additional control, a cross between C2 and the F⁻ parent showed no R^+ colonies among 50,000 $T^+L^+S^r$ progeny examined (Table 2). We conclude that the R^+ colonies found in the crosses involving these $R1^-$ strains were derived from genetic recombinants and, therefore, that there exist several closely linked genetic sites at which an $R1^-$ mutation can occur.

In Table 2 are also reported crosses of $R2^-$ mutants with the F⁻ parent. The proportion of R^+ recombinants ranged from 60% to 68%, which is consistent with the previous conclusion that $R2^-$ mutations are transferred later than the T^+ and L^+ genes.

The location of the $R1$ region was determined by timing the transfer of an $R1^-$ mutation to an F⁻ mating partner. The results in Fig. 1 show that transfer of the $R1$

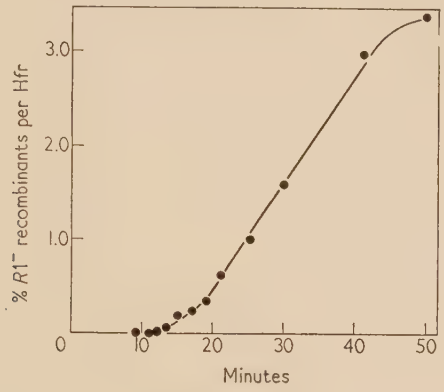

FIG. 1. Kinetics of transfer of the $R1$ marker by Hfr strain K10. The parental strains for the cross were Hfr $R1^-$ $P^+T^+L^+S^s$ (mutant C2) and F⁻ $R^+P^+T^+L^+S^r$. The ordinate scale is the interval between the time at which the parental cultures were mixed together (time zero) and the time at which the sample was blended to interrupt mating. Overnight cultures in P medium (with growth factors added as required) were subcultured in P medium and aerated until the density of the Hfr strain was 1×10^8/ml. and the F^- strain was 2×10^8/ml. The F⁻ culture was centrifuged and resuspended in one-fifth the initial volume of P medium. Equal volumes of the Hfr and F⁻ cultures were mixed together and shaken gently at 35°C. At the times indicated in each experiment, a 0·1 ml. portion was removed and diluted into 1·9 ml. of cold 0·1 M-tris buffer, pH 7·5. The diluted samples were immediately blended for 30 sec in a Lourdes Mixer and plated for recombinants.

region begins at about 13 minutes after the parental cultures are mixed. The times of transfer of other genetic markers by the Hfr strain are as follows: P (the structural gene for alkaline phosphatase) and *Lac y* (the permease gene for β-galactosidase) beginning at about 13 min and T and L beginning at about 21 min.

Since the results of the blendor experiments indicated close linkage between the $R1$ and P regions, the extent of this linkage in recombination units was measured in a cross between an $R1^-P^+$ donor and an $R1^+P^-$ recipient. Among the selected $T^+L^+S^r$ progeny, 1·8% $R1^+P^+$ recombinants were found. (The reciprocal recombinant type $R1^-P^-$ was not scored.) This map length is about tenfold greater than the length of the structural gene for alkaline phosphatase (Levinthal, 1959; Garen, 1960). The $R1$ and P regions are, therefore, closely linked, but it remains to be determined whether or not they are contiguous.

The third series of crosses involved the $R2$ group of constitutive mutations. The Hfr strain chosen for these crosses (Hfr 1) transfers the $R2$ region earlier than the TL

region (cf. Fig. 2 described below). The behaviour of the $R2^+$ region of the Hfr strain was examined in a cross with an F^- carrying an $R2^-$ mutation. Among the selected $T^+L^+S^r$ progeny, 20% were R^+. This value is lower than expected, in view of the fact that the $R2$ region is transferred earlier than the selected TL region. The low value may be due to a difficulty in pairing of donor and recipient chromosomes, since the strains were not made isogenic.

Independently isolated constitutive mutants of Hfr 1 were screened to determine if they were in the $R1$ or $R2$ groups. The classification was based on the proportion of R^+ recombinants obtained in crosses against an F^- strain carrying an $R2^-$ mutation. The results of crosses with fifteen constitutive mutants are shown in Table 3. It is possible

TABLE 3

Crosses of R^- *mutants of strain Hfr 1 against an* $R2^-$ F^- *strain*
Hfr $R^-P^+T^+L^+S^s \times F^-$ $R2^-P^+T^-L^-S^r$ (strain C4 F3)

Hfr parent	Number of $T^+L^+S^r$ recombinants tested	R^+ recombinants	Percent R^+ among $T^+L^+S^r$
C10	461	6	1·3
C11	1345	19	1·4
C12	949	26	2·8
C13	894	137	15·5
C14	692	7	1·0
C15	1348	24	1·8
C16	1260	22	1·7
C17	1399	252	18·0
C18	1141	18	1·6
C19	2032	13	0·64
C20	1856	314	17·0
C21	1598	47	2·9
C22	1495	17	1·1
C23	1813	18	1·0
C24	1964	308	15·7

The procedure for these crosses was identical to the one described in Table 2. The F^- parent, C4 F3, contains the $R2^-$ mutation of the mutant C4 of Hfr strain K10.

to arrange the mutants in two groups, with recombination values ranging from 0·64% to 2·9% in one, and from 15·5% to 18% in the other. These results suggest that the first group is composed of $R2^-$ mutants, and the second group of $R1^-$ mutants.

This conclusion is supported by the results of two other crosses. In one cross, two of the mutations in Hfr 1 that were classified as $R2^-$ (cf. Table 3) were mapped against each other. The Hfr parent was C23 and the F^- parent contained the constitutive mutation from C21. The proportion of R^+ recombinants found was 1·0% ($14R^+$ among 1469 $T^+L^+S^r$ colonies tested). This is in the range reported in Table 3 for $R2^-$ mutations in Hfr 1 mapped against an $R2^-$ mutation of K10, and therefore indicates that $R2^-$ mutations in strains K10 and Hfr 1 are located in corresponding regions of the genetic map. In the other cross, an $R1^-$ mutation in K10 was mapped against an $R1^-$ mutation of Hfr 1. The Hfr parent was mutant C2 of K10 and the F^- parent contained the mutation from mutant C17 of Hfr 1. Among a total of 7640 $T^+L^+S^r$ progeny tested, 5 were found to be R^+. This low proportion of R^+ recombinants indicates that $R1^-$ mutations in K10 and Hfr 1 are also located in corresponding regions of the map.

The location of the *R*2 region in Hfr 1 was determined by the timing experiment shown in Fig. 2. Transfer of the *R*2 region began at 7 min after the parental cultures were mixed. A separate experiment showed that the *TL* region was transferred beginning at 24 min. The *TL* region can now be used as a reference point to coordinate the locations of the *R*1 and *R*2 regions. From experiments with K10 we know that transfer of the *R*1 region begins at 13 min in this strain (cf. Fig. 1), and the *TL* region at 21 min. Therefore, the distance between the *R*1 and *R*2 regions, in units of time of transfer from donor to recipient, is 25 min.

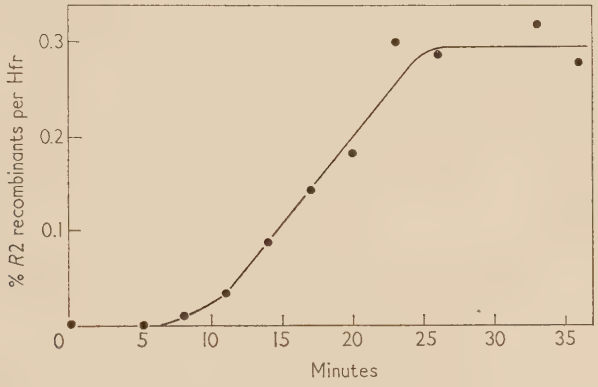

FIG. 2. Kinetics of transfer of the *R*2 marker by strain Hfr 1. The parental strains for the cross were Hfr $R2^{-}P^{+}T^{+}L^{+}S^{s}$ (mutant C21) and F^{-} $R^{+}P^{+}T^{+}L^{+}S^{r}$. The procedure for the experiment was the same as for Fig. 1.

In conclusion, the foregoing experiments have shown that all of the constitutive mutations are located in two regions of the map, which we designate as the *R*1 and *R*2 genes. Each gene comprises a closely-linked cluster of mutable sites. A cell containing a mutation in either gene is phenotypically constitutive.

(b) *Synthesis of alkaline phosphatase in heterozygotes*: cis-trans *tests*

The properties of partial zygotes, in which either the *R*1 or *R*2 gene is present in a heterozygous state, have been examined in the following experiments. The point in question is whether the R^{-} alleles are dominant or recessive in the *cis* and *trans* positions relative to the P^{+} gene.

(i) Trans *test on the* R1 *gene*

The first experiment involved $R1^{+}P^{+}/R1^{-}P^{-}R2^{+}$ heterozygotes formed in a cross between an $R1^{+}P^{+}R2^{+}$ donor and an $R1^{-}P^{-}R2^{+}$ recipient. Strain K10 was used as the donor because it transfers the *R*1 and *P* genes early (cf. Fig. 1—it should be noted that, since the *R*1 and *P* genes are closely linked and therefore transferred at about the same time, most of the zygotes studied in this cross were diploid for both the *R*1 and *P* genes) The results in Fig. 3 show that there is no increase in enzymatic activity when the zygotes are incubated in excess phosphate (repressing) medium, while in limiting phosphate medium activity begins to increase at about 40 min. We conclude that the $R1^{-}$ allele is recessive in the *trans* position relative to the P^{+} gene.

As a control experiment to follow constitutive enzyme synthesis in *trans* heterozygotes, a cross was run between K10 and an $R1^{+}P^{-}R2^{-}$ recipient. Since the *R*2 gene is transferred by K10 with less efficiency than the *R*1 and *P* genes, the major class of zygotes

that receive the P^+ gene in this cross have the genotype $R1^+P^+/R1^+P^-R2^-$ and are, therefore, phenotypically constitutive. The procedure was the same as in the preceding cross (Fig. 3). It was found that there was an increase in enzymatic activity already at time zero (i.e. 30 min after the parental cultures had been mated), and that the activity continued to rise throughout the 140 min period of the experiment. Since these results prove that constitutive enzyme synthesis is detectable in zygotes, the failure to detect constitutive synthesis in the preceding cross (Fig. 3) can be considered as significant.

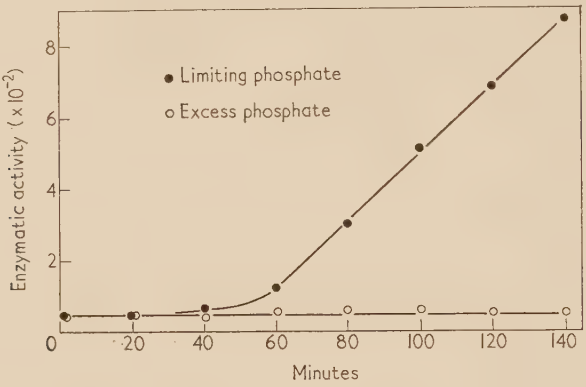

FIG. 3. Synthesis of alkaline phosphatase in $R1^+P^+/R1^-P^-$ zygotes. The parental strains for the cross were Hfr $R1^-R2^+P^+S^s$ (strain K10) and F$^-$ $R1^-R2^+P^-S^r$ (strain C2 F8 which carries the $R1^-$ mutation from C2). The procedure for the experiment was as follows. Overnight cultures of the Hfr and F$^-$ strains in P medium were diluted in P medium to yield a concentration of approximately 4×10^7 cells/ml. The cultures were aerated at 37°C and, when the cell densities reached approximately 2×10^8/ml., the Hfr and F$^-$ cultures were mixed together to give a ratio of Hfr to F$^-$ of approximately 2. The mixed culture was shaken gently in a 37°C bath for 30 min, at which time the cells were centrifuged, washed in 0·1 M-tris buffer, pH 7·4, and resuspended in S medium with 0·25% glucose. The resuspended culture was divided into two portions; to one was added limiting phosphate ($1{\cdot}6 \times 10^{-5}$ M-KH_2PO_4), and to the other excess phosphate ($6{\cdot}4 \times 10^{-4}$ M-KH_2PO_4). Streptomycin was added immediately to both portions at a concentration of 200 mg/l. to block enzyme synthesis by the Hfr cells, and the cultures were aerated at 37°C. Time zero in the figure is the time at which the streptomycin was added.

Samples of the mated cultures were prepared for enzyme assays by adding 1·0 ml. portions to 0·1 ml. of 0·1% merthiolate (to stop enzyme synthesis). The cells were centrifuged, washed and resuspended in 0·1 M-tris buffer, pH 7·4. A drop of toluene was added to the cell suspensions and they were then shaken at 37°C for 60 min. Enzyme assays were performed as described in the text.

Furthermore, the results explain why the preceding cross showed a lag of 40 min before enzymatic activity began to increase in limiting phosphate medium; since the lag is not observed with a phenotypically constitutive heterozygote, it can be attributed to the time required for establishing conditions of phosphate starvation.

(ii) Cis *test on the* R*1 gene*

In the next two experiments, the behaviour of heterozygotes that carried the $R1^-$ gene in the *cis* position relative to the P^+ gene was examined. In one experiment, $R1^-P^+/R1^+P^-R2^+$ heterozygotes were produced in a cross between $R1^-P^+R2^+$ and $R1^+P^-R2^+$ parents. The results in Fig. 4 show that in excess phosphate medium there is no increase in enzymatic activity, whereas in limiting phosphate medium activity begins to increase at about 40 min. (The high background in this cross is due to the activity initially present in the constitutive Hfr parent.) As an additional control, a

cross was run with the constitutive Hfr parent and an $R1^{-}P^{-}R2^{+}$ recipient, producing $R1^{-}P^{+}/R1^{-}P^{-}R2^{+}$ constitutive zygotes. In this cross, the activity began to increase in excess phosphate at about 40 min. We conclude that the $R1$ allele is recessive in the *cis* position relative to the P^{+} gene.

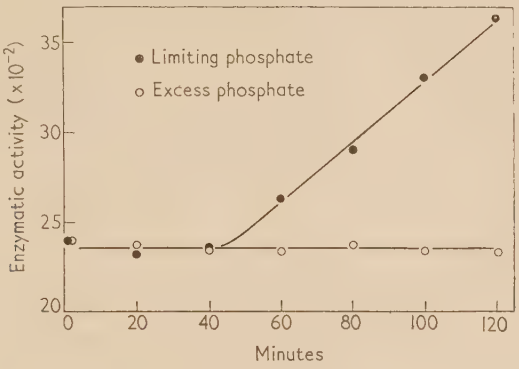

FIG. 4. Synthesis of alkaline phosphatase in $R1^{-}P^{+}/R1^{+}P^{-}$ zygotes. The parental strains for the cross were Hfr $R1^{-}R2^{+}P^{+}S^{s}$ (mutant C2) and F^{-} $R1^{+}R2^{+}P^{-}S^{r}$ (a strain which carries the P^{-} mutation U13). The procedure for the experiment was the same as for Fig. 3.

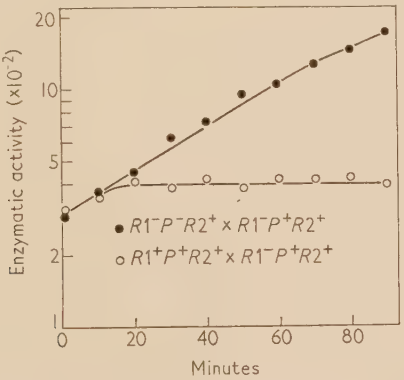

FIG. 5. Synthesis of alkaline phosphatase in $R1^{+}P^{+}/R1^{-}P^{+}$ zygotes. The parental strains for the cross in which $R1^{+}P^{+}/R1^{-}P^{+}$ zygotes were produced (open circles) were $F'R1^{+}R2^{+}P^{-}S^{s}$ (which transfers the $R1$ and P markers) and F$^{-}R1^{-}R2^{+}P^{+}S^{r}$ (an F^{-} strain which contains the $R1^{-}$ mutation from the Hfr mutant C2). In the control cross (filled circles) the parental strains were Hfr $R1^{-}R2^{+}$ $P^{-}S^{s}$ (a P^{-} mutant derived from the constitutive mutant C2 of strain K10) and the same F^{-} strain used above; this control cross measures the rate of constitutive enzyme synthesis in $R1^{-}$ cells under mating conditions. The procedure for the experiments was as follows. Overnight cultures were subcultured in Penassay medium (which contains excess phosphate) and aerated at 37°C. When the density reached approximately 3×10^{8} cells/ml. the parental cultures were mixed together (time zero in the Figure) at a ratio of 2×10^{8} F' cells/ml. to 2×10^{7} F^{-} cells/ml. and the culture was shaken gently in a 37°C water bath. The culture was diluted at 40 min twofold with Penassay medium and streptomycin was added at 42 min at a concentration of 100 mg/l. to stop the growth of the F' parent. Samples were prepared for enzyme assay as described in Fig. 3. The enzymatic activities measured after 40 min were multipled by a factor of two to compensate for the dilution.

This conclusion was confirmed by a second *cis* experiment with $R1^{+}P^{+}/R1^{-}P^{+}R2^{+}$ heterozygotes. The donor was an F' strain that transfers an extrachromosomal segment carrying $R1^{+}$ and P^{+} genes, and the recipient was $R1^{-}$ $P^{+}R2^{+}$. The results are shown in Fig. 5. Within a period of 20 min after mixing the parental cells, constitutive enzyme synthesis was completely blocked.

It has also been possible to study the behaviour of *cis* heterozygotes by means of the F' progeny formed in the preceding cross. Two hours after the parental cells were mixed, the culture was plated on TGSE agar (the streptomycin in the agar prevented growth of the F' parental cells). The resulting colonies were tested with NPP, and it was found that 95% were phenotypically R^+. These colonies presumably contained $R1^+P^+/R1^-P^+$ $R2^+$ heterozygote F' cells. The heterozygote character of the cells in one of the R^+ colonies was verified by the following test. A culture prepared from a single cell was plated on TGSE agar, and the resulting colonies were tested with NPP. About 0·5% were found to be phenotypically R^-. This shows that the original R^+ cell contained an R^- allele which segregated occasionally during growth.

(iii) Cis *test on the* R2 *gene*

The foregoing experiments have demonstrated the recessive character of an $R1^-$ gene in *cis* and *trans* heterozygotes. We have next examined the behaviour of heterozygotes for the $R2$ gene, first with the $R2^-$ allele in the *cis* position, $R2^+/R2^-R1^+P^+$. The heterozygotes were formed in a cross between an $R2^+R1^+P^+$ donor and an $R2^-R1^+P^+$ recipient. Strain Hfr 1 was used for this cross because it transfers the $R2$ marker early (cf. Fig. 2). The results are shown in Fig. 6. The constitutive synthesis of alkaline

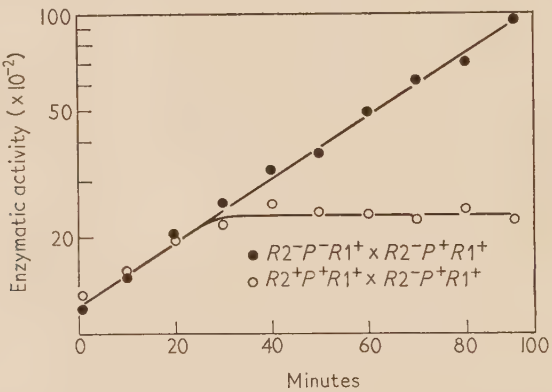

FIG. 6. Synthesis of alkaline phosphatase in $R2^+/R2^-R1^+P^+$ zygotes. The parental strains for the cross in which $R2^+/R2^-R1^+P^+$ zygotes were produced (open circles) were Hfr $R2^+R1^+P^+S^s$ (strain Hfr 1) and F$^-$ $R2^-R1^+P^+S^r$ (an F$^-$ strain which contains the $R2^-$ mutation from the Hfr mutant C4). In the control cross (filled circles) the parental strains were Hfr $R2^-R1^+P^-S^s$ (a P^- mutant derived from the constitutive mutant C23 of strain Hfr 1) and the same F$^-$ strain used above; this control cross measures the rate of constitutive enzyme synthesis in $R2^-$ cells under mating conditions. The procedure for the experiment was the same as for Fig. 5.

phosphatase by the recipient cells stopped completely within 30 min after addition of the donor cells, which is approximately the time required for completion of the transfer of the $R2^+$ gene. We conclude that the $R2^-$ gene is recessive in a *cis* position relative to the P^+ gene.

(iv) Trans *test on the* R2 *gene*

The *trans* experiment for the $R2$ gene was done by crossing an $R2^-P^-R1^+$ mutant of Hfr 1 with an $R2^+P^+R1^+$ recipient, under the same conditions used in the *cis* experiment. There was no increase in enzymatic activity in excess phosphate medium over a period of two hours. This result is consistent with the recessive character of the $R2^-$ gene shown in the *cis* experiment.

(c) *Amounts of alkaline phosphatase activity in constitutive mutants*

The extent to which a constitutive mutation is able to overcome repression of alkaline phosphatase synthesis was determined by comparing the enzymatic activity of a culture grown with excess phosphate to the activity in limiting phosphate. The results obtained with both $R1^-$ and $R2^-$ mutants are shown in Table 4. We first consider the

TABLE 4

Alkaline phosphatase activity of constitutive mutants

Strain	*R* genotype	Enzymatic activity/cell density	
		excess phosphate	limiting phosphate
Hfr 1	R^+	0·0	8·6
K10	R^+	0·0	8·4
C2	$R1^-$	2·4	3·2
C3	$R1^-$	5·3	10·3
C5	$R1^-$	2·4	3·1
C7	$R1^-$	2·0	2·9
C8	$R1^-$	2·5	3·3
C9	$R1^-$	2·8	3·3
C13	$R1^-$	2·9	3·4
C27	$R1^-$	3·0	3·6
C28	$R1^-$	2·9	3·8
C10	$R2^-$	11	14
C11	$R2^-$	10	11
C12	$R2^-$	12	13
C14	$R2^-$	7	12
C15	$R2^-$	11	12
C18	$R2^-$	10	13
C19	$R2^-$	11	13
C23	$R2^-$	10	11
C31	$R2^-$	10	12

The cultures were grown to maximum cell density in S medium containing a limiting concentration of glucose (0·025%) and excess phosphate, and were diluted 100-fold in two media: S medium containing limiting glucose (0·025%) and excess phosphate, and S medium containing excess glucose (0·25%) and limiting phosphate (5×10^{-5} M-KH_2PO_4). The subcultures were grown to maximum cell density at 37°C with aeration; approximately the same maximum density was reached in both kinds of media. The optical density at 540 mμ was read on each culture, and a sample was prepared for enzyme assay by centrifuging and resuspending the cells in 0·1 M-tris buffer, pH 7·4 and shaking the cell suspension with toluene. Enzyme assays were performed as described in the text.

behaviour of the nine $R2^-$ mutants. It should be noted that the activities of the $R2^-$ mutants in limiting phosphate averaged 12 units, which was greater than the average value of 8·5 units for the parental R^+ cultures. This difference is to be expected, since an R^- culture is able to synthesize enzyme throughout the growth period, while an R^+ culture begins to synthesize enzyme at the terminal stage of growth, when phosphate limitation has been established. The activity of each of the $R2^-$ mutants in excess phosphate was close to, but consistently below, its activity in limiting phosphate. This suggests that the $R2^-$ mutants are slightly sensitive to repression.

We next consider the behaviour of the nine $R1^-$ mutants. For eight of the mutants, enzymatic activities in limiting phosphate ranged from 2·9 to 3·8, in contrast to the

average value of 12 which was found for the $R2^-$ mutants. In excess phosphate, the activities were from 15 to 30% below the values in limiting phosphate. The remaining $R1^-$ mutant, C3, differed from the other $R1^-$ mutants in that its activity in limiting phosphate had the high value characteristic of the $R2^-$ mutants.

The data in Table 4 have shown an important difference between $R1^-$ and $R2^-$ mutants, namely, that most of the $R1^-$ mutants produce only about one-fourth the alkaline phosphatase activity of the $R2^-$ mutants, in limiting phosphate as well as in excess phosphate. Thus, an $R1^-$ mutation can cause two phenotypic changes: the mutant cell becomes capable of synthesizing the enzyme constitutively, but the maximum amount of enzymatic activity that can be produced (as shown by the results in limiting phosphate) is abnormally low.

It is possible that the reduced level of alkaline phosphatase activity in $R1^-$ mutants may be part of a more general inhibitory effect on the activities of many enzymes. In order to test for this effect, we have compared the amount of induced β-galactosidase activity produced by the $R1^-$ mutant C8 to the activities produced by an $R2^-$ mutant and by the parental R^+ strain. For this comparison, the cells were grown to phosphate limitation, since this condition was employed in measuring the maximum level of alkaline phosphatase activity (cf. Table 4). The results reported in Table 5 show that the three

TABLE 5

Synthesis of β-galactosidase under conditions of phosphate limitation

Strain	*R* genotype	Enzymatic activity/cell density	
		β-galactosidase	alkaline phosphatase
C8	$R1^-R2^+$	4·2	3·0
C4	$R1^+R2^-$	3·8	9·4
K10	$R1^-R2^+$	4·0	7·8

The cultures were grown to maximum cell density in S medium containing a limiting concentration of sodium lactate (0·02%). Excess lactate (0·4%) was subsequently added; the phosphate concentration in the medium (5×10^{-5} M-KH_2PO_4) was the limiting factor in the presence of the excess lactate. The cultures were aerated at 37°C, and when a cell density was reached at which phosphate limitation became established (as judged by the onset of phosphatase synthesis), 10^{-3} M thiomethylgalactoside was added as an inducer for β-galactosidase. After 24 hr aeration, the cell density was read and the cultures were centrifuged, resuspended in 0·1 M-tris buffer, pH 7·4 and shaken with toluene for 15 min at 37°C. Enzymatic assays were carried out on the toluene-treated suspensions.

strains produced approximately equal amounts of β-galactosidase activity. Thus, the inhibitory effect of $R1^-$ mutations on alkaline phosphatase activity appears to be a specific effect on this enzyme.

(d) *Screening for operator-type constitutive mutations*

Constitutive β-galactosidase mutations which are dominant in *cis* heterozygotes have been described in *E. coli* (cf. Introduction). They have been interpreted as affecting an operator gene that controls the site of action of a repressor. The operator gene for β-galactosidase is closely linked to the structural gene, *z*, and the regulator gene, *i*.

We have looked for operator-type mutations for alkaline phosphatase by the following method. Strain $F'13$ is homozygous diploid for a segment which includes both the P and the $R1$ genes, but not the $R2$ genes ($R1^+P^+/R1^+P^+R2^+$). In order for a constitutive mutation for alkaline phosphatase to be expressed in this strain, it must be either an operator mutation (i.e. expressed in the heterozygous state) or an $R2^-$ mutation; the probability of finding an $R1^-$ mutant of $F'13$ should be negligibly low, since two mutations would be required to achieve a homozygous $R1^-$ diploid. Fifty independently-occurring constitutive mutants were isolated from $F'13$ by the same selective procedure used with Hfr strains (Torriani & Rothman, 1961). The mutants were mated with a repressible F^- strain, and the cultures then were plated on TGSE agar. When the resulting colonies were tested with NPP there were no constitutive colonies found among several hundred colonies examined from each cross. This indicates that the parental F' constitutive mutants did not have operator mutations that could be transferred to an F^- mating partner. A negative result does not mean that operator mutations do not exist for alkaline phosphatase, since they might have been missed by the screening procedure for the following reasons: the mutation frequency might be very low relative to $R2^-$ mutations; the operator gene might not be closely linked to the P gene and therefore not be present on the extrachromosomal segment of the F' strain; operator mutations for alkaline phosphatase might affect the structure of the enzyme, producing a P^- phenotype.

4. Discussion

The results we have reported demonstrate a basic similarity between the mechanisms for repression of alkaline phosphatase and β-galactosidase, namely that repression appears to be mediated by a cytoplasmic agent (repressor) whose formation is genetically controlled (regulator gene). This is shown by the recessive character of constitutive mutations in *cis-trans* tests on heterozygotes which carry the constitutive mutation in either the *cis* or *trans* positions relative to the active structural gene (cf. Introduction and experiments in Figs. 3, 4, 5, and 6).

There are also several points of difference that emerge from a comparison of the regulating systems for alkaline phosphatase and β-galactosidase. One difference is in the number of regulator genes. There is a single regulator gene for β-galactosidase (Pardee *et al.*, 1959), and there are two separate regulator genes for alkaline phosphatase. (The existence of two unlinked genes controlling the induced synthesis of tyrosinase in *Neurospora* has been reported recently—Horowitz, Fling, Macleod & Sueoka, 1960.)

A second difference is in the relative rates of phenotypic expression of a regulator gene and a structural gene in heterozygotes. In the β-galactosidase system, when the i^+ (regulator) and z^+ (structural) genes of a donor are crossed into an i^-z^- recipient, a period of about two hours ensues during which constitutive enzyme synthesis occurs, which indicates that the repressor is formed at a slower initial rate than is the enzyme (Pardee *et al.*, 1959). For the alkaline phosphatase system, in a comparable experiment in which the $R1^+$ and P^+ genes of a donor are crossed into an $R1^-P^-$ recipient (cf. Fig. 3), there is no constitutive synthesis of the enzyme, which indicates that the repressor is formed at least as rapidly as the enzyme.

A third difference is in the effect of constitutive mutations on the maximum amount of enzymatic activity that can be produced under *non-repressed* growth conditions. The i^- constitutive mutants for β-galactosidase produce as much enzymatic activity as the i^+ strain after induction (Cohen-Bazire & Jolit, 1953). Similarly, the $R2^-$ mutants for

alkaline phosphatase produce as much enzymatic activity in limiting phosphate as the R^+ strain. Most of the $R1^-$ mutants, however, produce an abnormally low amount of enzymatic activity in limiting phosphate, as compared to the $R2^-$ and R^+ strains.†

We are indebted to Dr. C. Levinthal for many valuable discussions about the experiments.

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† There have recently been isolated constitutive mutants for β-galactosidase which produce a low level of enzyme both in the presence or absence of added inducer (Monod & Jacob, personal communication). These mutants may be similar to the $R1a^-$ mutants for alkaline phosphatase.

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Control of Arginine Biosynthesis in Strains of *Escherichia coli* not Repressible by Arginine†‡

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The efficiency of control of arginine biosynthesis has been compared in three strains of *Escherichia coli*: strain W, in which the enzymes of this pathway are repressible by arginine; strain B, in which these enzymes are not repressible by arginine and are maintained at a low level; and a mutant of B, strain B4S–7, in which these enzymes are not repressible by arginine but are maintained at a high level. The control was measured by observing the incorporation of a limited supply of ^{14}C-arginine, in competition with endogenous arginine, under steady-state conditions in a chemostat.

It was found that the ability of arginine to repress enzyme synthesis is not essential for controlling arginine synthesis, but the level of the enzymes is critical. Arginine synthesis was controlled well only in the strains in which the level of the enzymes was kept low. Repression, which appears to control enzyme levels in most strains, thus plays an important role not only in the economy of protein formation but also in the control of metabolite biosynthesis. *E. coli* B has an alternative mechanism for limiting enzyme levels, and its presence may provide an explanation for the natural selection of such a non-repressible strain.

Evidence is presented that feedback inhibition in the arginine pathway acts on the first specific enzymatic step leading to arginine formation, the conversion of glutamate to N-acetylglutamate.

1. Introduction

Two mechanisms are known by which an end-product can influence its own biosynthesis: feedback inhibition of enzyme activity and repression of enzyme formation. One can infer from theoretical considerations that feedback inhibition alone is necessary and sufficient for the control of end-product biosynthesis. In its presence, repression would appear to be merely a mechanism for economy in protein synthesis, stopping the formation of enzymes when they are not needed.

In order to verify this point one must vary the two mechanisms independently. In previous experiments (Gorini, 1958) a discrimination was attempted by adding various amounts of arginine to a steady-state culture of *E. coli* strain W growing exponentially in minimal medium (i.e. under conditions of partial repression). It was found that as long as the amount of added arginine did not exceed that quantity which would be endogenously synthesized, no further repression of ornithine

† A preliminary report of part of this work has been published (*Fed. Proc.* **18**, 222, 1959).

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transcarbamylase (an essential enzyme in arginine biosynthesis)† occurred. This result led to the inference that feedback inhibition continuously adjusts arginine biosynthesis to that amount which exactly complements the exogenous supply, and that this adjustment can occur within a range of intracellular arginine concentrations that does not affect the degree of repression. However, these experiments did not explore the role of repression in controlling arginine biosynthesis, since the enzyme levels were always lower than they would have been without repression. It is conceivable that at the high level of enzymes characteristic of de-repression, the intracellular arginine might not have been so effective in controlling its own formation.

This problem has now been investigated by comparing feedback control in 3 strains of *E. coli*: strain W repressible by arginine and two strains that are both non-repressible by arginine but that have different levels of the enzymes of the arginine pathway. In one non-repressible strain, wild type B, the level of the enzymes of arginine biosynthesis (enzymes 3 to 7 were analysed) is low, comparable to that found in a repressible strain growing exponentially in minimal medium. In the other (B4S-7), isolated as a spontaneous mutant of strain B, the level of the enzymes assayed (enzymes 3, 4, 5 and 7) is high, comparable to that found in repressible strain W under conditions of de-repression.

The existence in strain B of a feedback inhibition loop in the arginine pathway has already been indicated by previous isotopic experiments (Abelson, 1954) on the kinetics of the competition of exogenous arginine for incorporation with that synthesized endogenously. Enzyme inhibition was postulated to account for the observed immediate preferential utilization of exogenous arginine upon addition of an excess of this compound to a growing culture of strain B. The subsequent discovery (Gorini, unpublished) that strain B is not repressible by arginine further strengthened this hypothesis.

The purpose of the present investigation is to explore the efficiency of the feedback control of arginine biosynthesis in the aforementioned 3 strains under conditions where the exogenous supply can provide only a fraction of the arginine required.

The results have made it possible to obtain an understanding of the biological significance of repression for the control of end-product formation, and furthermore have offered an explanation for the successful competition of a non-repressible strain in natural selection.

This work also furnished evidence for the location of the enzymatic step in the arginine pathway the activity of which is inhibited by arginine.

2. Materials and Methods

(a) *Culture medium*

Medium A (Davis & Mingioli, 1950), a mineral salt-citrate medium, was employed in all experiments. Glucose as source of carbon, L-glutamic acid, N-acetyl-L-glutamic acid, and uniformly labeled ^{14}C-L-arginine (obtained from Nuclear Chicago) were added as sterile solutions as indicated in each experiment.

† A schematic outline of the biosynthesis of arginine by *E. coli* is given below. The numbers refer to the enzymes that catalyze each reaction.

$$\text{glutamate} \xrightarrow{1} \text{N-acetylglutamate} \xrightarrow{2} \text{N-acetylglutamic semialdehyde} \xrightarrow{3} \text{N-acetylornithine} \xrightarrow{4}$$
$$\text{ornithine} \xrightarrow{5} \text{citrulline} \xrightarrow{6} \text{argininosuccinate} \xrightarrow{7} \text{arginine}.$$

(b) *Chemostat culture conditions*

Steady-state cultures were obtained by growing the different arginine prototrophs in chemostats (Novick & Szilard, 1950) with a generation time of 2 to 3 hr. The amount of growth, usually controlled by limiting the glucose, was maintained at a level equal to that reached by an arginine auxotroph limited by 10 μg/ml. arginine. The auxotrophs corresponding to the 3 strains were individually tested because differences in growth response to arginine were observed. This means that the prototrophs are synthesizing an amount of arginine equivalent to 10 μg/ml. of culture. Different amounts of uniformly labeled arginine (100 cts/min/μg) were added to the chemostats after equilibration (4 to 5 generations) and samples were taken for analysis after 4 or 5 more generations.

(c) *Measurement of radioactivity in cell protein and culture fluid*

For determining the radioactivity incorporated into protein, samples of culture were mixed with an equal volume of 10% trichloroacetic acid, placed in a boiling water bath for 30 min, cooled in an ice bath, and filtered and washed on a membrane filter. The radioactivity in the culture fluid was determined on samples of culture passed through a membrane filter. The radioactivity of the fractions soluble in cold and in hot trichloroacetic acid was found to be only 1 to 2% of the total and so was ignored.

Ten to twenty percent of the radioactivity, depending on the strain used, remained in the medium even when the organism was supplied with only small amounts of ^{14}C-arginine. The culture supernatants were analysed to determine the identity of this radioactivity. It was not associated with arginine, since the supernatant was unable to support any growth of an arginine auxotroph. On paper chromatography the radioactivity moved with agmatine and was provisionally identified with this decarboxylation product of arginine. Furthermore, the total counts obtained showed a deficit corresponding to the amount of decarboxylation calculated on the basis of agmatine found. Corrections for this loss have been made in preparing Fig. 1.

Radioactivity was measured employing a Nuclear-Chicago model D47 gas-flow counter with micromil window.

(d) *Measurement of bacterial density*

Bacterial density was measured by optical density in a Beckmann DU spectrophotometer at 490 mμ using a 1 cm cuvette. An optical density of 1·000 corresponds to about 1 mg dry weight bacteria per ml.

(e) *Enzyme essays*

The enzymes were determined in samples of the culture treated with toluene. Ornithine transcarbamylase (enzyme 5) was assayed as described previously (Gorini, 1958), and argininosuccinase (enzyme 7) according to the method of Ratner (1952). One unit of enzyme forms one micromole of product per hour. Specific activity is given as the units per mg dry weight of bacteria.

(f) *Origin and properties of bacterial strains employed*

E. coli W, a wild type, is repressible by arginine for all the enzymes (enzymes 3 to 7) of arginine synthesis that have been tested (Vogel, 1956; Albrecht & Vogel, 1960; Gorini & Maas, 1958). *E. coli* B, another wild type, is non-repressible by arginine for these enzymes. *E. coli* B4S-7, a spontaneous mutant isolated from strain B (Gorini & Gundersen, in manuscript), is non-repressible like B, but has a much higher level of the enzymes of arginine biosynthesis that were tested. Strain B77-23 is a prototroph permeable to acetylglutamate. To obtain it, several arginine auxotrophs isolated from strain B were first screened on plates of minimal medium containing acetylglutamate and no arginine. One strain gave colonies which could grow when inoculated on minimal medium supplemented with acetylglutamate or arginine but not on minimal medium alone. From such a secondary mutant (B77), a back mutant to prototrophy was isolated by selection on a minimal

medium plate. This strain, B77–23, is still permeable to acetylglutamate as shown in a later section. The B77–23 histidine auxotroph and all the arginine auxotrophs used were blocked in positions other than enzyme 5 and were obtained by ultraviolet irradiation and penicillin selection (Gorini & Kaufman, 1960). The arginine requirer, W45A–25, used in the syntrophism experiment, was blocked between citrulline and arginine.

3. Experimental

(a) *Enzyme levels in the strains used*

Table 1 gives the specific activity of enzymes 5 and 7 in strains W, B, and B4S-7 grown (1) in the presence of an excess of arginine, (2) in minimal medium in the absence of exogenous arginine, and (3) under conditions in which arginine limits the rate of growth (using arginine auxotrophs derived from these strains). It is seen that the level of the enzymes in condition (2) is the same in strains W and B and is 15 to 30 times higher in strain B4S-7, approaching the derepressed level (condition (3)) of strain W.

TABLE 1

The effect of arginine on the level of enzyme 5 and enzyme 7 in various strains of E. coli

Strain	Excess arginine†		Minimal medium‡		Limiting arginine §	
	enzyme 5	enzyme 7	enzyme 5	enzyme 7	enzyme 5	enzyme 7
	units		units		units	
W	0·1	0·1	6·0	0·2	104·0	1·3 ¶
B	7·0	0·4	5·0	0·2	4·0	—
B4S-7	160·0	1·5	132·0	1·6	140·0	—

† Overnight culture grown in minimal medium plus arginine (100 μg/ml.).

‡ Overnight culture grown in minimal medium.

§ Arginine auxotrophs of the corresponding strains grown in chemostats under arginine limitation.

¶ This value was obtained by culturing a slow-growing prototroph (D22) which possesses a defect in arginine synthesis making arginine limit the growth in minimal medium. (This situation is equivalent to an internal chemostat.)

(b) *Influence of enzyme level on the efficiency of feedback inhibition*

The outline of the experiments is basically the same as that described in a previous paper (Gorini, 1958). Cultures of prototrophs were maintained at a given bacterial density by limiting glucose in a chemostat, and were provided with varying amounts of arginine in the medium. In the previous work using only repressible strains, the essentially complete utilization of exogenous arginine was inferred from the absence of repression of enzyme 5. In the present experiments, since this index of utilization could not be employed with non-repressible strains, ^{14}C-arginine was used to permit direct determination of its incorporation.

Figure 1 confirms, by these more direct measurements, the previous conclusions concerning the utilization of exogenous arginine by repressible strain W: the added compound, up to an amount just adequate for complete replacement of the endogenous supply, was all incorporated. Identical behavior was shown by strain B, which is non-repressible but has a moderate level of arginine-synthesizing enzymes.

In contrast, strain B4S-7, which has a high level of these enzymes, exhibited less complete incorporation of exogenous arginine. It is evident, by comparison between the theoretical curves for perfect control and for no control of arginine synthesis, that this strain has an intermediate degree of control.

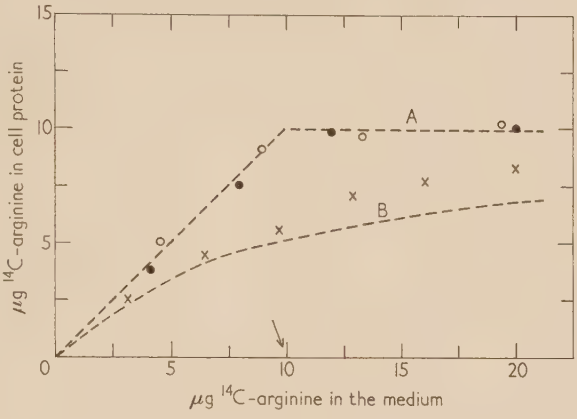

FIG. 1. Incorporation of ^{14}C-arginine into cell protein by steady-state cultures of *E. coli*.

A—Theoretical curve calculated on the basis of complete preferential utilization of up to 10 μg/ml. exogenous ^{14}C-arginine.

B—Theoretical curve for simple dilution of exogenously supplied ^{14}C-arginine calculated on the basis of dilution with 10 μg/ml. of endogenously synthesized arginine.

Different amounts of uniformly labeled ^{14}C-arginine (100 cts/min/μg) were added to different steady-state cultures of the three organisms growing in chemostats. The bacterial density was controlled by limiting glucose. The points on the curve are those corrected to the values which would be obtained when the bacterial density is equal to that reached by the corresponding arginine auxotroph growing on 10 μg/ml. arginine.

● Strain W ○ Strain B × Strain B4S-7

Table 2 gives the level of enzyme 5 in these strains in the various steady-state conditions of this experiment. Strain W exhibited, as previously observed (Gorini, 1958), an abrupt change in the level of repression when the amount of added arginine exceeded that which could be incorporated.† Strain B showed a constant low level

TABLE 2

Level of enzyme 5 in steady-state cultures of E. coli *strains supplied exogenously with different amounts of* ^{14}*C-arginine*

μg^{14}C-arginine/ml. provided in chemostat	Units of enzyme 5 (specific activity)		
	E. coli W	*E. coli* B	*E. coli* B4S-7
0	8·5	3·7	190
4	8·7	3·2	204
8	8·5	4·1	185
12	0·1	3·5	186
16	—	—	211
20	0·1	4·7	172

The experiment is described in the legend for Fig. 1.

† The implications of this behavior for the relative sensitivity of the mechanisms of repression and feedback inhibition have been extensively discussed elsewhere (Gorini, 1958).

of the enzyme, and strain B4S-7 a constant high level, similar to those of Table 1. In Table 2, enzyme 5 is taken as an example; a similar pattern was also observed with enzyme 7. This pattern can reasonably be predicted for the other enzymes of the pathway, and in particular for that under feedback inhibition. It would follow that in strain B4S-7, the excessive level of the inhibitable enzyme would be associated with an increased steady-state level of intracellular arginine. Further observations confirmed this prediction by showing that this strain, in contrast to strains B and W, excretes arginine. The excretion was demonstrated by tests for syntrophism on solid minimal medium, streaking each prototrophic strain next to a streak of a mutant (W45A-25) blocked immediately before arginine.

(c) *Site of feedback inhibition*

The efficient control of arginine biosynthesis in a strain non-repressible by arginine offers a simple way to determine at which enzymatic step feedback inhibition is exerted, for with such a culture one should be able to demonstrate excretion of arginine by providing the product of the reaction under feedback inhibition. It has been shown for other pathways (Yates & Pardee, 1956; Umbarger & Brown, 1958) that feedback inhibition is exerted at the first enzyme specific to the pathway. In the arginine pathway, by analogy, the inhibited enzyme should be acetyl-glutamate synthetase (reaction 1). However, though syntrophism experiments with strain B showed excretion of arginine when the medium was provided with citrulline or ornithine, there was no excretion with acetylglutamate. Since it was possible that, in contrast with previous observations in *E. coli* (Vogel, Abelson & Bolton, 1953), the strain B used was not sufficiently permeable to acetylglutamate, a substrain (B77-23) was isolated following a procedure (see Materials and Methods (f)) which should yield a permeable strain. This strain was found to excrete arginine when acetylglutamate or any other arginine intermediate was provided in the medium.

This behavior supports the hypothesis that enzyme 1 is the step subject to feedback inhibition. However, a more rigorous experiment has been performed in the chemostat using a histidine auxotroph of strain B77-23 with histidine limiting growth and using procedures similar to those described for Fig. 1. Different ^{12}C-precursors of

Table 3

Incorporation of exogenous ^{14}C-arginine into protein by steady-state cultures of B77–23 hist$^-$

Chemostat I		Chemostat II	
Glutamate added (μg/ml.)	^{14}C-arginine in protein (μg/ml. culture)	Acetylglutamate added (μg/ml.)	^{14}C-arginine in protein (μg/ml. culture)
0	5·3	0	5·1
35	5·4	35	1·7

Two identical chemostats were set up. Growth medium: medium A plus 4 μg/ml. histidine and 7 μg/ml. ^{14}C-arginine. This amount of histidine allows a level of growth corresponding to that reached by an arginine auxotroph growing on 14 μg/ml. arginine. Samples were taken for analysis as described in Materials and Methods, then 35 μg/ml. of glutamate or acetylglutamate were added, respectively, to the medium of chemostats I and II and samples were taken again after equilibration.

arginine (glucose, glutamate, or acetylglutamate) were tested for their ability to compete with exogenous ^{14}C-arginine for incorporation into protein. The results presented in Table 3 show that with acetylglutamate, in contrast to glucose or glutamate, the feedback control of arginine biosynthesis is lost: arginine inhibits enzyme 1, and by adding acetylglutamate one by-passes this reaction. An alternate hypothesis, that arginine may inhibit enzyme 2 and that acetylglutamate reverses this inhibition by substrate competition, is not rigorously disproved, but it is unlikely because of the low molar ratio of acetylglutamate/arginine (about 2) that proved sufficient in this experiment. In the feedback inhibition of *iso*leucine biosynthesis, for example, the inhibitory effect of *iso*leucine on the action of the first enzyme of the sequence could be reversed by the substrate of the enzyme, but only in a molar ratio of 100 to 1.

4. Discussion

Feedback control not only enables a cell to spare the synthesis of building blocks when they are available in the surrounding medium; it also permits a cell growing without an exogenous supply to adjust continuously the rate of synthesis of a building block to its utilization for the synthesis of macromolecules. By studying the effect of limited supplies of exogenous arginine, it was possible to reproduce conditions approximating those which exist in a cell continuously adjusting the rate of building block synthesis. Using this approach the present investigation compares the efficiency of the control of arginine biosynthesis of 2 strains non-repressible by arginine (B and B4S-7) and one repressible by arginine (W).

The behavior of the 2 wild type strains, repressible W and non-repressible B, is very similar. The results show that both strains when grown in minimal medium possess a similar level of enzymes involved in arginine biosynthesis and are able to control with equal efficiency the rate of formation of this end-product. In this respect neither strain has a selective advantage over the other, possibly accounting for the existence of both strains as wild types. In strain B4S-7, in contrast, where the level of enzymes is high, the control of arginine biosynthesis is inefficient.

A detailed genetic analysis of these strains will be published elsewhere (Gorini & Gundersen, in manuscript) but a brief survey is pertinent here. The level of arginine enzymes in the 2 wild type strains is determined by quite different factors. In strain W it is controlled by the repression exerted by endogenous arginine; while in strain B though there is no repressibility, by arginine, some other mechanism prevents the arginine enzymes from reaching a level as high as in de-repressed strain W. This mechanism could involve discrete mutations, one for each individual enzyme of the sequence. However, the fortuitous isolation from B of mutant B4S-7, still non-repressible but with a very high level of arginine enzymes 3, 4, 5 and 7, makes this hypothesis untenable; for a single mutational event has affected the level of at least four enzymes. This finding strengthens the idea that the sequence of arginine enzymes in strain B is under a kind of control different from repressibility by arginine, and that in B4S-7 this control is absent. This would imply that the level of the enzyme(s) under feedback inhibition is also low in B and high in B4S-7.

This predicted high level of the enzyme under feedback control in strain B4S-7 could account for the inefficient control of arginine synthesis in this strain. The higher the level of this enzyme, the higher would be the concentration of intracellular

arginine required for adequate feedback; and there are obvious limits to the level of intracellular arginine compatible with quantitative uptake of exogenous arginine.

In vivo experiments reported in this paper indicate that the enzyme under feedback inhibition is acetylglutamate synthetase, the first of the sequence. Although its existence has been recognized (Maas, Novelli & Lipmann, 1953) the level of this enzyme is only inferred in this discussion because it has not proved possible to measure it reliably.

The genetic origin of B4S-7 which is a not infrequent type of spontaneous mutant derived from B probably by a one-step mutation, makes it unlikely that B4S-7 differs from B by more than one character. Since the mutation from B to B4S-7 already involves the control of enzyme level, the sensitivity of the molecules of acetylglutamate synthetase to arginine inhibition in B4S-7 is in all probability the same as in B. Nevertheless, the control of arginine biosynthesis is less efficient in B4S-7 than in B (or W). One may conclude that feedback inhibition alone is not sufficient to control arginine biosynthesis efficiently: the level of the enzymes of the sequence must also be kept sufficiently low in some independent way, either by repression, or by the presence of another controlling genetic factor. Thus repression, which did not seem necessary for fine control of end-product biosynthesis, can actually share with feedback inhibition the responsibility for that control.

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The Reaction of Tobacco Mosaic Virus Ribonucleic Acid with Hydroxylamine

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The pyrimidine bases of tobacco mosaic virus RNA react with hydroxylamine under rather mild conditions. By using the nucleosides and nucleotides of the corresponding bases the reaction mechanism with hydroxylamine was clarified. Uridine was split into ribosyl-urea and 5-*iso*xazolone. Cytidine only added hydroxylamine presumably to the C_4—C_5 double bond, readily converting the C_6 amino-group to the keto-group.

In the RNA cytosine at pH 6 reacted with hydroxylamine at least 30 times faster than uracil did, whereas at pH 9 uracil reacted at least 8 times faster than cytosine. Thus the reaction could be controlled to give the rather specific base elimination or alteration without splitting the polynucleotide chain. The applications of the findings for studies with infectious nucleic acid are discussed.

1. Introduction

Two papers published in previous years have dealt with the action of hydroxylamine on viruses. Kozloff, Lute & Henderson (1957) presented data showing that hydroxylamine inactivates certain bacteriophages, probably by splitting the thiolester bonds in their protein component. Franklin & Wecker (1959) succeeded in inactivating various RNA-containing animal viruses, as well as the infectious RNA of the mouse encephalomyelitis virus, with hydroxylamine. No chemical evidence for a reaction of hydroxylamine with the RNA was presented although it was shown that only the RNAs of the viruses and not their protein components were the point of attack of the hydroxylamine. The authors did not succeed in inactivating Newcastle disease and mumps viruses although they too contain RNA. Therefore they presumed that a specific component of RNA, not present in all virus RNAs, reacted with hydroxylamine. The hypothesis was presented that an amino acid was attached to the high molecular weight virus RNA by an ester bond similar to that found in soluble RNA. If this hypothesis were true the amino acyl group would then be responsible for the infectivity of the virus RNA and it should be possible to split it off by hydroxylamine.

Later Schuster & Franklin (1959, unpublished results) showed that the infectious RNA of TMV† could also be inactivated by hydroxylamine. Simultaneously they presented evidence that the inactivation was not due to the removal of an amino acid bound to the RNA. Therefore only a reaction of hydroxylamine with the nucleic acid itself could be the cause of the observed inactivation.

† The following abbreviations are used: TMV = tobacco mosaic virus, C = cytidine, Cp = cytidine-2′,3′-phosphate, U = uridine, Up = uridine-2′,3′-phosphate, 5-HMC = 5-hydroxymethyl cytosine, *p*-DAB = *p*-dimethylaminobenzaldehyde.

The mutagenic action of hydroxylamine in addition to the lethal action has been demonstrated (Schuster & Wittmann, 1961). It therefore appeared worthwhile to study the chemical reactions of hydroxylamine in more detail. In the present paper the specific reaction of hydroxylamine with the pyrimidine bases of nucleic acid is reported.

2. Materials and Methods

A-grade nucleic acid derivatives (bases, nucleosides and nucleotides) from California Corporation for Biochemical Research were used.

TMV RNA was prepared by the phenol method (Schuster, Schramm & Zillig, 1956; Gierer & Schramm, 1956). Calf thymus DNA and T2 phage DNA were prepared according to Signer & Schwander (1949) and Wyatt & Cohen (1953) respectively.

Salt-free NH_2OH was prepared by treating NH_2OH,HCl (p.a. substance, Merck) with Dowex-1 (OH^- form) in water until the solution was approximately neutral. After the removal of the resin the solution was concentrated in a flash evaporator to a final concentration of approximately 2 M. The solution was kept frozen at — 15°C.

Ribosyl-urea was prepared by the following method analogous to the condensation of urea derivatives with sugars (Erickson & Keps, 1953). Urea (0·025 mole) was condensed with ribose (0·025 mole) in 40 ml. 95% ethanol and 0·45 ml. conc. HCl for several days at 50°C. Samples of this solution were chromatographed in several systems and ribosyl-urea was determined by analysis for urea and ribose as described below.

The synthesis of 5-*iso*xazolone was achieved as follows: Ethyl sodioformylacetate, $NaOCH{=}CH{-}COOC_2H_5$, was prepared according to Wislicenus (1887). The ethyl ester was crystallized from water-free ethanol and incubated in alkaline solution with an excess of NH_2OH,HCl for 45 min at 70°C, a reaction analogous to that used in the preparation of 3-methyl-5-*iso*xazolone by Hantsch (1891). Samples of this solution were tested chromatographically (see below).

Ribose-oxime was prepared by treating ribose (5 mg/ml.) with an excess of aqueous salt-free NH_2OH at pH 7·5 and 20°C for several days. This solution was used directly for paper chromatography.

Urea derivatives were determined qualitatively by their condensation with phenyl hydrazine to the corresponding diphenyl carbazides, which gave violet colored inner complex salts with Ni compounds. These salts are readily soluble in chloroform (Feigl, 1960). On paper chromatograms the urea derivatives were identified by the yellow color produced after spraying with an alcoholic solution of *p*-DAB (Fink, Cline, McGaughey & Fink, 1956).

Ribose and ribose derivatives were located on paper chromatograms by spraying with aniline phthalate (Partridge, 1949). Nucleosides with acid stable N-glycosidic bonds cannot be demonstrated by aniline phthalate, but these compounds form white spots on a dark blue background after spraying the chromatogram with a solution of $NaIO_4$ and benzidine (Gordon, Thornburg & Werum, 1956).

Compounds that couple with diazotized sulfanilic acid (for example *iso*xazolones) were located on paper chromatograms by spraying with a solution of 4 M-$NaNO_2$, glacial acetic acid and 1% sulfanilic acid in 25% acetic acid in the proportion 1 : 1 : 1 (by vol.), which had reacted for 30 min to form the diazo-sulfanilic acid. The chromatograms were then sprayed with 10 M-NaOH, whereupon the compounds yielded red-orange zones.

Hydroxylamine was determined by its oxidation to nitrous acid in the presence of sulfanilic acid, the sulfanilic acid being thereby diazotized, and afterwards coupled with α-napthylamine to form a red dye (Feigl, 1960). The quantitative determination of hydroxylamine was based on this reaction. The extinction of the red dye was measured at 520 mμ. The extinction of 2 μg NH_2OH/ml. was 1·8 with a light path of 1 cm.

Ribose was determined by a slightly modified orcinol method (Dische & Schwarz, 1937). The extinction of 10 μg ribose/ml. was 1·0 at 760 mμ with a 1 cm light-path.

Fehling solution was prepared according to Feigl (1960). Nitrogen was determined according to the micro-Kjeldahl method and phosphorus according to Martin & Doty (1949).

For chromatography the following systems were used:

aqueous butanol 86% (v/v);
collidine, water saturated;
butanol: acetic acid: water 5 : 1 : 4 (by vol.);
ethanol-water 7 : 3 (v/v);
butanol-ethanol-5 M-HCl, 3 : 2 : 2 (by vol.);
acetone-25% trichloroacetic acid, 3 : 1 (v/v).

(a) *Spectrophotometric investigation of the reaction of hydroxylamine with purines and pyrimidines*

The compounds were dissolved in 0·02 M-phosphate buffer pH 7 and the pH was further adjusted by addition of acid or base. The concentration was chosen so that the maximum absorption of the solution was between 1·7 and 2·2 with a light path of 1 cm (solution 1). Hydroxylamine hydrochloride was dissolved in water and the pH brought to the same value as solution 1 by alkali (solution 2). Usually the final concentration of solution 2 was 2 M. First two quartz cuvettes were filled with solution 1 and solution 2, and one placed behind the other in the spectrophotometer so that the light passed through both cuvettes (optical mixture). Then, the two solutions were mixed, and 2 cuvettes filled and placed in the spectrophotometer (chemical mixture). The absorption of the chemical mixture was compared with the optical mixture.

(b) *Reaction of nucleic acids with hydroxylamine*

The nucleic acids were dissolved in NH_2OH,HCl, the pH of which was adjusted by addition of alkali. The following conditions were used for incubation:

1. TMV RNA (final concentration 2 mg/ml.) in 1 M-NH_2OH,HCl at 20°C, up to 300 hr incubation.
2. calf thymus DNA (final concentration 1·5 mg/ml.) in 0·8 M-NH_2OH,HCl at 20°C up to 280 hr, and
3. T2 phage DNA (final concentration 1 mg/ml.) in 2 M-NH_2OH,HCl at 37°C up to 300 hr.

After treatment the solutions were dialysed against water to remove the hydroxylamine. The RNA was hydrolysed by alkali and the nucleotides determined. The DNA was hydrolysed by formic acid and the bases also determined.

3. Results

(a) *Spectrophotometric investigation of the reaction of hydroxylamine with nucleic acids and their constituents*

No decrease in molecular weight was found after treating liver RNA with hydroxylamine for 48 hours (Franklin & Wecker, 1959). Poliomyelitis virus RNA which has lost its infectivity completely after treatment with 0·1 M-hydroxylamine at pH 7 also showed no decrease in molecular weight (Holland, McLaren, Hoyer & Syverton, 1960). It appeared, therefore, that the RNA sugar-phosphate backbone had not been split by hydroxylamine. If, on the other hand, hydroxylamine reacted with the bases of the nucleic acid a spectral change would probably be demonstrable. To test this hypothesis several bases and their nucleosides and nucleotides, were treated with hydroxylamine and their spectra measured during the treatment. Unexpectedly the u.v. absorption of the pyrimidines, cytosine and uracil and their derivatives decreased slowly whereas the purines showed no alteration. From Table 1, where the percentage decrease of u.v. absorption of several compounds under identical conditions is compared, the following conclusions can be drawn. Pyrimidines without substituents on positions 4 or 5 show a large decrease in absorption. Pyrimidines with substituents on these C-atoms react slightly or, as is the case with thymidine, only under more drastic conditions. Whereas

the decrease in absorption of TMV RNA is small in comparison to U or C, it must be considered that the extinction of the pyrimidines in TMV RNA amounts only to one third of the total absorption. The real decrease in absorption is three times the measured value. Even if this correction is made the pyrimidines in a polynucleotide chain react slowly in comparison to the pyrimidine nucleosides.

Table 1

Decrease of u.v. absorption of pyrimidine derivatives and nucleic acids by treatment with hydroxylamine

Compound	Wavelength (mμ)	% Decrease in absorption
Uracil	260	19
Uridine	260	27
Thymidylic acid	270	< 1
Orotic acid	280	< 4
Cytosine	265	20
Cytidine	270	12
5-Methylcytosine	270	< 3
4-Aminocytosine	260	< 1
Adenylic acid	260	0
Guanylic acid	250	0
TMV RNA	260	2
Thymidine	265	15

With the exception of thymidine, reactions were carried out in 1 M-NH_2OH, pH 7·5 for 15 hr at 20°C. Thymidine was treated with 2 M-NH_2OH, pH 7·5 for 15 hr at 45°C.

In addition the decrease in absorption during treatment of C and U with hydroxylamine was measured as a function of pH. C reacts more rapidly at high H-ion concentrations than at low concentrations, whereas U shows the reverse tendency. Therefore it is assumed that C reacts in a different way from U (Table 2).

Table 2

The pH dependence of decrease in u.v. absorption of the pyrimidines after treatment with 1 M-NH_2OH at 20°C for 15 hours (decrease in % of the starting amount)

pH	4·75	6·2	7·1	7·5	8·0	9·15	9·6
Cytidine	11	45		12		3	
Uridine	1	15	25		27		14

(b) *Evidence of the reaction of hydroxylamine with the nucleic acids by chemical analysis*

In order to confirm the changes of the pyrimidines found by spectral measurements by chemical analysis, TMV RNA was treated with hydroxylamine at different pH values and samples were withdrawn from time to time to determine the nucleotide composition. The velocity of the decrease of the pyrimidine bases in the RNA was obtained in this

manner. Cytosine in the RNA reacted more rapidly at pH 6·15 than at pH 9·15, whereas uracil again showed the reverse tendency (Table 3). Thus nearly the same pH dependence of the reaction rates of the pyrimidine bases was obtained by chemical and spectrophotometrical analyses.

TABLE 3

Decrease of the pyrimidine bases in TMV RNA after treatment with 1 M hydroxylamine for 150 hours at 20°C

RNA base	pH of the solution	Decrease of the RNA base (%)†
Cytosine	6·15	32
	7·55	13
	9·15	< 4
Uracil	6·15	< 1
	7·55	13
	9·15	30

† The base content of untreated RNA = 100%.

Thymus DNA and the 5-HMC-containing DNA of phage T2 were treated with hydroxylamine in order to determine the reactivity of pyrimidines substituted in position 5. A base analysis showed only a decrease of cytosine and 5-HMC, in the treated DNA in comparison to the untreated controls, but not of thymine. Cytosine and 5-HMC both reacted somewhat slower than cytosine or RNA.

The resistance of the purine bases in RNA to hydroxylamine had to be proved. During the treatment of the RNA with hydroxylamine at pH 6·15 and pH 9·15 the amounts of adenine and guanine were determined relative to the amount of RNA phosphorus. The values obtained were constant independent of the time of treatment, indicating the non-reactivity of the purine bases.

(c) *The reaction of the model substances U and Up with hydroxylamine*

In order to elucidate the reaction mechanism of hydroxylamine with the pyrimidine bases of RNA, U and Up or C and Cp, were used as model substances. RNA itself is not a convenient model substance because it contains both pyrimidines and purines.

Uridine was dissolved in 1 M-hydroxylamine (pH 7·5) and, after one week of treatment at 20°C, chromatographed in aqueous butanol. Only uridine was detectable on the chromatogram by u.v. light. To identify possible reaction products of uridine which showed no u.v. absorption, the chromatogram was cut into several strips and sprayed with aniline phthalate, diazo sulfanilic acid, $NaIO_4$-benzidine, and *p*-DAB (see Methods). Up to 5 new compounds were detectable which must have arisen by the reaction of hydroxylamine with U, since U and hydroxylamine showed none of these compounds when tested separately.

The time dependence of the formation of these substances was investigated in order to obtain an insight into the single steps of the reaction. Uridine was therefore treated with hydroxylamine for 2, 3, 5, 6, and 8 days and then chromatographed in aqueous butanol. Two compounds were isolated as the first reaction products. They could not be transformed into each other and were identified as ribosyl-urea and 5-*iso*xazolone.

Ribose oxime was identified as a third reaction product and as a degradation product when ribosyl-urea was treated with hydroxylamine. The R_F values and the spot tests of the substances mentioned above are summarized in Table 4. Their exact identification was carried out after elution from the chromatogram.

TABLE 4

Characterization of the compounds, obtained by treatment of uridine with hydroxylamine at pH 7·5 and 20°C

Number	Compound	R_F-value in aqueous butanol relative to U	Identification†
I	Ribosyl-urea	0·38	yellow color with p-DAB, brown color with aniline phthalate, white spot with $NaIO_4$-benzidine.
II	5-*Iso*xazolone	0·48	orange-red dye formed by coupling with diazo-sulfanilic acid, ($NaIO_4$-benzidine reaction not unequivocal).
III	Ribose oxime	0·84	brown color with aniline phthalate, white spot with $NaIO_4$-benzidine.

† See under Methods also.

The following reactions provide evidence that compound I is ribosyl-urea. (1) It reduces Fehling solution. (2) It contains a ureido-group detectable by condensation with phenyl hydrazine (cf. Methods). (3) The compound was not adsorbed by Dowex-1 resin and the molar ratio N/ribose was 1·76 after passing through an exchange column in order to remove N-containing impurities. (4) It is very unstable, ribose being already detectable as a degradation product by chromatography in aqueous butanol. (5) Ribosyl-urea synthesized by acid condensation of ribose and urea possessed the same R_F value as I in aqueous butanol and in aqueous collidine. The ureido-group was also detectable by phenyl hydrazine in the synthesized product.

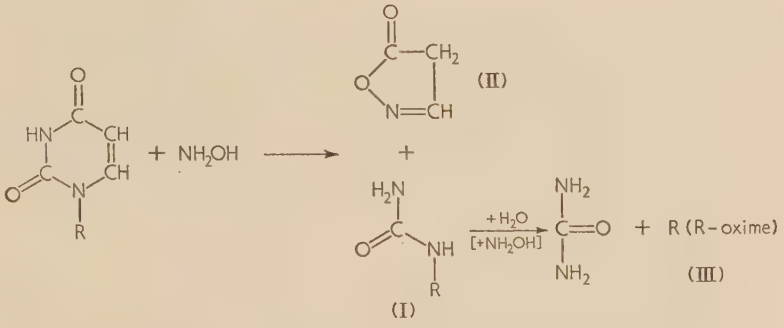
A comparison of the chromatographic behaviour of compound II and synthesized 5-*iso*xazolone was carried out. Both R_F values were identical in aqueous butanol. Since evidence was obtained for the splitting of the uracil base by identification of compound I, it was not thought necessary to characterize compound II exactly. Furthermore in the case of the reaction of uracil in RNA with hydroxylamine compound II is removed from the chain.

Compound II was also identified when Up was treated with hydroxylamine at pH 7·5. Instead of compound I, ureido-ribosyl-2′-3′-phosphate was found, which does not move in the aqueous butanol system and was detected by spraying with aniline phthalate.

The ribose content of the assumed ribose oxime was determined by the orcinol method. Heating in 2 M-HCl for 15 min at 100°C split off 0·85 mole hydroxylamine per mole ribose from compound III. The R_F values of compound III and synthesized ribose oxime were identical in the following systems: aqeuous butanol; butanol : acetic acid; ethanol : water.

To make sure that uridine was also split in weak alkaline solution by hydroxylamine it was treated at pH 9·2 with hydroxylamine. Ribosyl-urea and ribose oxime were also detected chromatographically as degradation products under these conditions.

Summarizing the data on the reaction products the following mechanism of splitting of uridine and uridine-2′-3′-phosphate by hydroxylamine is proposed: (R — ribose and ribose-2′-3′-phosphate, respectively).



In one experiment compounds I and III were determined quantitatively by the orcinol reaction after their chromatographic separation. Simultaneously the decrease of uridine was determined spectrophotometrically. 84 to 92% of the uridine that had disappeared was found as ribosyl-urea and ribose oxime, thus providing further evidence that equation I represents the principal if not the only reaction of U with hydroxylamine.

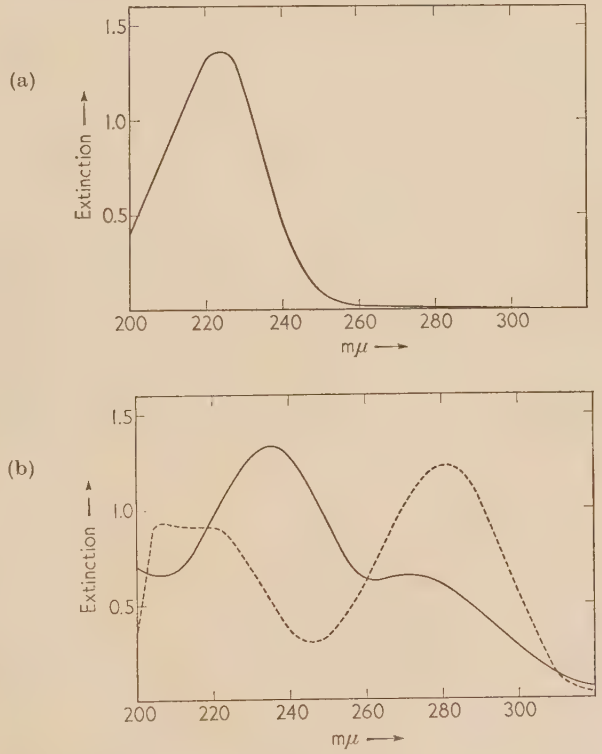


FIG. 1. The u.v. absorption spectra of the reaction products of cytidine with hydroxylamine.

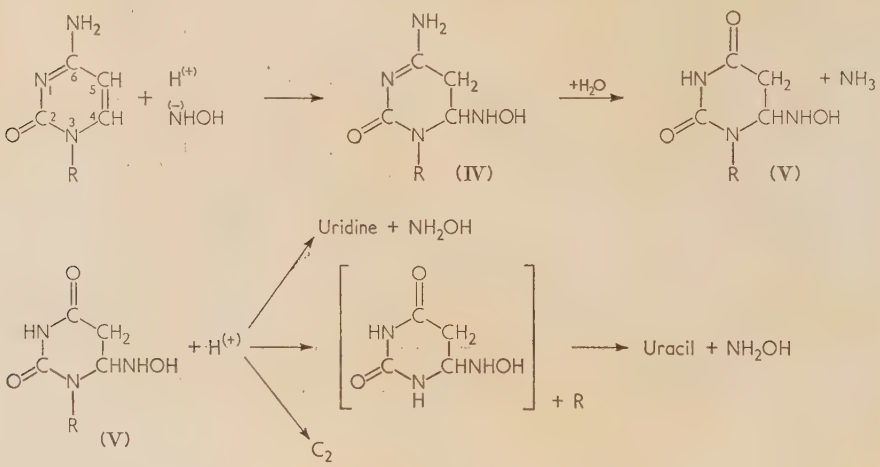
(a) Full line, C_1 at neutral pH.
(b) Full line, C_2 at neutral pH;
Pecked line, C_2 in 1 M-HCl.

(d) *The reaction of the model substances C and Cp with hydroxylamine*

C was treated in 1 M-hydroxylamine, pH 6·2 at 20°C for several days and chromatographed in aqueous butanol. In contrast to the hydroxylamine treated U, two new u.v. absorbing substances were found and designated C_1 and C_2 (Plate I). Neither ribosyl-urea nor ribose and ribose oxime, were detectable on the chromatogram. Therefore the reaction of C with hydroxylamine must follow a mechanism different from that of U. The u.v. spectra of C_1 and C_2 are shown in Fig. 1.

The spectrum of C_1 is characterized by the absence of the maximum of C at 270 mμ. Only a maximum at 225 mμ was found which is probably due to the chromophore $O{=}C_2{-}N_1{=}C_6{-}$ in the altered cytosine molecule. Generally in the case of the pyrimidines saturation of the double bond at C_4 produces loss of u.v. absorption in the region of the maximum near 260 mμ, whereas the second maximum in the region of 225 mμ is retained or increased (Shugar, 1960). Therefore it is supposed that in C_1 hydroxylamine is added to the $C_4{-}C_5$ double bond. This addition should lead to structure IV (see equation 2), since C_4 is electropositive in comparison to C_5 and hydroxylamine reacts as a nucleophilic agent. The finding that substitution on C_4 or C_5 inhibits the addition of hydroxylamine supports this structure (see Table 1).

When C_1 was treated with 1 M-HCl for 20 min at 100°C uridine, uracil, ribose, hydroxylamine, and C_2 were found, but C was obviously not re-formed (Plate I(b)). Therefore, the following is proposed for the formation and degradation of C_1: (R = ribose).



The following reasons favour structure V instead of IV for C_1. At neutral pH a deamination of dihydrocytidine to dihydrouridine takes place very easily during the hydrogenation of C (Green & Cohen, 1958). Furthermore, the addition product of hydroxylamine with Cp corresponding to C_1 has a N/P ratio of 3 (see below) corresponding to V (R = ribose-2′, 3′-phosphate). A compound with a N/P ratio of 4 corresponding to IV was never found.

Compound V might be expected to split into *iso*xazolone (II) and ribosyl- urea (I
A compound which coupled with diazo-sulfanilic acid, and which possessed the same R_F value as II in aqueous butanol, was found only after treating C_1 with 1 M-HCl for 20 min at 100°C. Therefore, it is unlikely that V is an intermediate during the formation

(a)

PLATE I. The chromatographic behaviour of the reaction products of cytidine with hydroxylamine. (a) C_1 and C_2 in aqueous butanol.

[*To face page* 454

PLATE I. The chromatographic behaviour of the reaction products of cytidine with hydroxylamine. (b) C_1 treated in HCl (1), and warmed 23 hr at 37°C in neutral solution (2). Aqueous butanol chromatogram.

of II from the reaction of U with hydroxylamine at neutral pH and likely that hydroxylamine attacks U at the C_6-position.

Compound C_1 was unstable in alkaline solution (1 M-KOH for 18 hr at 20°C) and its u.v. absorption was lost. This may be the reason why a u.v. absorbing reaction product of C and Cp respectively could not be found after alkaline hydrolysis of RNA treated with hydroxylamine. C_1 splits off ribose, when warmed for 23 hr at 37°C, and a u.v. absorbing compound remains under these conditions (Plate I(b)).

Compound C_2 was not characterized in more detail. It appeared whenever re-chromatography of C_1 was attempted and also upon treating C_1 with HCl (see equation 2). C_2 is therefore presumed to be a secondary reaction product of C_1 and not a primary reaction product of C and hydroxylamine. In contrast to C_1 the ribose in C_2 was stable to acid treatment. C_2 did not reduce Fehling solution.

The reaction of cytidylic acid (Cp) with hydroxylamine was analogous to that of C with hydroxylamine. Two u.v. absorbing compounds were also obtained (designated Cp_1 and Cp_2). Their spectra were nearly identical with that of C_1 and C_2. Cp_1 and Cp_2 were separable from one another in the acetone: CCl_3COOH system and both were exchangeable on the anion exchanger, Dowex-1. After elution from the resin with 0·01 M-HCl, both compounds showed a N/P ratio of 3 (see above).

By treating Cp_1 with 1 M-HCl for 20 min at 100°C hydroxylamine was split off (0·5 mole NH_2OH to 3 moles N and 1 mole P, respectively). Thereby Cp_1 was transformed into Up and Cp_2, but not into uracil, as would be expected from equation 2. Obviously phosphoric acid stabilizes the N-glycosidic bond. The structure V with R = ribose-2′-3′-phosphate is therefore assumed.

4. Discussion

The results demonstrate the specific action of hydroxylamine with the pyrimidine bases of the nucleic acid and simultaneously indicate that this reaction is also responsible for the inactivation of TMV RNA by hydroxylamine. Therefore the earlier hypothesis that amino acids attached to the RNA are necessary for the infectivity of the RNA can be rejected. If such amino acids actually occur in the RNA their splitting off by hydroxylamine has no influence on the infectivity of RNA.

The reaction of hydroxylamine with uridine is very similar to the reaction of hydrazine with uridine (Levene & Bass, 1927). In both cases the ureido-group $\diagdown N—CO—N\diagup$ is replaced by the configurations $\diagdown N—O—\diagup$ and $—N—N—$, respectively with the formation of *iso*xazolone and pyrazolones, respectively. In the case of cytosine both agents react differently. Hydrazine splits off the base with the formation of 3-amino-pyrazolone, whereas hydroxylamine is only added. But it should be mentioned that Cp is also more resistant to hydrazine than Up (Baron & Brown, 1955). Furthermore, it cannot be excluded that C is split by hydroxylamine under more drastic conditions, especially since the first reaction product of C with hydroxylamine is easily deaminated.

One can assume that the pyrimidine bases in the RNA react with hydroxylamine in the same way as the model substances chosen for the investigation. Uracil bases can be thereby eliminated in the polynucleotide chain without a splitting of the chain at these points. Only urea remains attached to the chain in a very labile glycosidic bond.

Cytosine bases are not eliminated but rather undergo a transformation which may be important for the biological activity of the RNA. By addition of hydroxylamine, the 6-amino group of cytosine is transformed to a keto group; thus cytosine is changed to a base analogous to uracil.

Furthermore, the reaction of hydroxylamine with RNA can be controlled to yield specific base elimination or alteration. In RNA cytosine reacts at pH 6·15 more than 30 times more rapidly with hydroxylamine than uracil, whereas at pH 9·15 uracil reacts at least 8 times more rapidly than cytosine (Table 3). Therefore it seemed interesting to look for mutations produced by such specific alterations. The mutagenic action of an uracil elimination as well as of a cytosine alteration could be demonstrated. This work is in preparation (Schuster & Wittmann, 1961).

I am indebted to Professor Schramm for discussions and generous support of the work and to Deutsche Forschungsgemeinschaft for financial support. The technical assistance of Mrs. D. Steffens is gratefully acknowledged.

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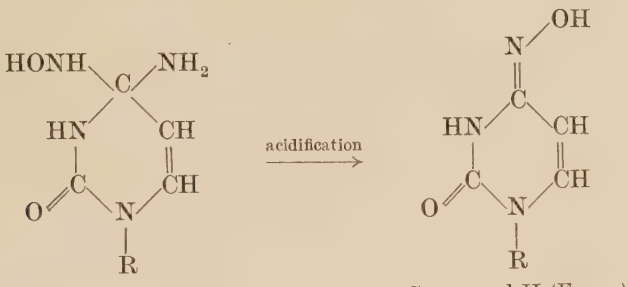
Note added in proof:

Freese, Bautz-Freese & Bautz (1961) have recently demonstrated the mutagenic action of hydroxylamine on T4 phage. These authors have also studied the reaction of hydroxylamine with model substances (Freese, Bautz & Bautz-Freese, 1961). Treating cytosine with hydroxylamine they isolated a compound I, which on acidification gives another compound II. The spectrum of the latter coresponds with the u.v.-absorption of the 6-ketoxime of uridine, i.e. 3-ribofuranosyl-6-hydroxylamino-2-pyrimidone (Fox *et al.*, 1959). Therefore the authors concluded that the initial attack

of hydroxylamine is at C_6 (see below) and not at C_4 of cytosine, as assumed in this paper.



C ompound I (Freese) Compound II (Freese)

Compounds I and II (Freese, Bautz & Bautz-Freese, 1961) probably correspond to compounds C_1 (Cp_1) and C_2 (Cp_2) in this paper, since the corresponding spectra agree well. Although we agree on the structure of C_2 (Compound II) some differences exist between the first reaction products, Compound I (Freese) and Cp_1 (this paper): Compound I should give a N/P ratio = 4 (with R = ribose phosphate) whereas Cp_1 has a N/P ratio = 3 and is converted not only to Cp_2 (6-ketoxine of uridylic acid) but also to uridylic acid on acidification (see above). Since Cp_2 is quite stable in acid solution uridylic acid must arise directly from Cp_1 during HCl treatment.

The conversion of Cp_1 to Cp_2 in acid solution must be accompanied by an intramolecular rearrangement, whereby hydroxylamine changes from C_4 to C_6 of the pyrimidine. This rearrangement, although sterically possible, has not been proved.

Summarizing all available data, still no definitive conclusions can be drawn regarding the initial point of attack of hydroxylamine on cytosine or its derivatives since in no case is the structure of the first reaction product proven.

I am much indebted to Professor Freese for sending his paper to me in advance of publication.

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A Relative Molecular Weight Series Derived from the Nucleic Acid of Bacteriophage T2 †

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Sedimentation velocity was measured for the following materials: (1) T2 DNA isolated without mechanical breakage and purified by ion-exchange chromatography; (2) the first breakage product produced by stirring T2 DNA; (3) subfractions of (2) separated by chromatography, including putative half molecules; and (4) the quarter-length analogues to (2) and (3) produced by stirring half molecules. Some of these materials were also studied by capillary viscometry and by density gradient centrifugation. Results are summarized in Table 1 and Fig. 6.

The results establish a relation between relative molecular weight, sedimentation coefficient, and viscosity over a fourfold range higher than that of DNA preparations studied previously. Quarter length fragments of T2 DNA are similar in physical properties to preparations of bacterial DNA to which a molecular weight of 16 million has been assigned. This and other facts set limits to the molecular weight of T2 DNA, which must lie between 50 and 120 million but cannot be further specified at this time.

1. Introduction

Measurements of molecular weights and molecular weight distributions of DNA present challenging physical problems and are often pertinent to questions about biological function. For the high molecular weight classes recently encountered in certain bacteria and bacteriophages, new methods of measurement will have to be found. In this paper we describe what we believe to be half molecules and quarter molecules derived by mechanical breakage of natural molecules of the DNA of T2, identified without resort to conventional measurements. We have not established absolute molecular weights for these materials, but we believe that such measurements will be possible and that our materials will assist in their evaluation. In connection with our work, it may be recalled that mechanical breakage of DNA at moderate rates of shear was reported by Cavalieri & Rosenberg (1959), Davison (1959), and Rosenkranz & Bendich (1959). These authors noted that degradation as measured by lowering of sedimentation coefficient and in other ways tended to a limit dependent on rate of shear, and discussed briefly the hydrodynamics of breakage.

2. Methods

The preparation of DNA from T2, the chromatographic methods, the breakage of DNA by stirring, and the qualitative identification of half molecules and quarter molecules

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produced by stirring, have been described before (Mandell & Hershey, 1960; Hershey & Burgi, 1960). For breaking DNA in dilute solutions, we used one of two stirrers. One consisted of a pair of crossed razor blades (Virtis Co., Yonkers, N.Y.) rotated in a horizontal plane with their cutting edges trailing. The other consisted of a glass rod with rounded ends, 6·4 × 33 mm, rotated horizontally about its center. Both were spun in a (Virtis Co.) 50 ml. fluted, conical flask, usually containing 50 ml. of DNA diluted in 0·65 M-NaCl, 0·05 M-phosphate, pH 6·7. Stirring speeds were monitored with a revolution counter and, when critical, are reported as averages of measurements taken at frequent intervals.

DNA concentrations were measured photometrically in terms of the nominal extinction coefficient of 21·3 cm^2/mg at 260 mμ.

Viscosity measurements were made in a three-bulb capillary viscometer, capillary diameter 0·865 mm, length 289 cm, calibrated by measurements with glycerol and water. The flow times of water could be predicted within 10% from the dimensions of the apparatus. Shear rates and method of extrapolation to intrinsic viscosity are presented with the results.

Sedimentation coefficients were measured in a 30 mm cell containing an aluminum centerpiece and spun in a Spinco model E centrifuge equipped with an ultraviolet absorption optical system. Light-absorbing materials derived from the gaskets used to seal the centrifuge cell were removed by a brief preliminary run at 10,000 rev/min. The washed cell was filled through a 5 ml. syringe barrel fitted with a 22 gauge needle, pouring the solution into the top and allowing it to flow under gravity or, with CsCl solutions, under reduced pressure. The conditions of measurement were: DNA concentration 10 μg/ml., solvent 0·72 M-NaCl, 0·05 M-phosphate, pH 6·7 (to correspond to the solvent in which DNA elutes from our column), rotor temperature 22 to 23°C (maintained nearly constant during acceleration), rotor speed 31,000 rev/min for whole molecules, 36,000 for half molecules, and 40,000 for smaller materials. Boundaries were traced in a Joyce-Loebl densitometer. The measurements refer to the position in the boundary corresponding to the midpoint of the change in film density, designated the median S and expressed in Svedberg units, without correction for the viscosity or density of the solution.

The above conditions were chosen in view of the following observations. (1) The sedimentation velocity of T2 DNA proved independent of DNA concentration within experimental error between 5 and 40 μg/ml. (2) The sedimentation was unaffected by lowering the salt concentration to 0·1 or 0·2 M, or by substituting tris(2-amino-2-hydroxymethylpropane-1 : 3-diol) (pH 7·4) or citrate (pH 7·4) for phosphate. (3) The velocity constant was lower and less reproducible, and less strongly dependent on molecular weight, when measured in "epon" or "Kel-F" centerpieces than when measured in aluminum centerpieces. This anomaly virtually disappeared when the DNA was degraded to $S = 30$ or less. (4) The sedimentation coefficient of T2 DNA fell rapidly as the rotor speed was reduced below the range 30,000 to 40,000 rev/min. This anomaly was also peculiar to high molecular weight DNA. (5) A sample of DNA from *Escherichia coli*, supplied by Paul Doty and his colleagues and previously studied by them, exhibited under our conditions $S = 33$, not significantly different from their $S_{20,w}$ extrapolated to zero concentration.

According to our unpublished experiments, items (3) and (4) above are to be explained as consequences of convection that sets in at the lower speeds, and seems to be unavoidable at all speeds when plastic centerpieces are used. Sedimentation of low molecular weight DNA is not subject to convective disturbances in the same degree, perhaps because the boundary formed is sharper.

T2 DNA of high molecular weight shows, under the conditions specified above, an asymmetrical boundary with modal S near 57, median S = 60 to 65, and a long tail with S up to 100 or more. The lower molecular weight materials we studied showed symmetrical boundaries.

"Band width" molecular weights were measured by equilibrium sedimentation in cesium chloride solutions of density 1·7 (Meselson, Stahl & Vinograd, 1957). The 12 mm, 4°, Kel-F cells were spun at 27,690 rev/min for 3·5 to 4·0 days, and contained 0·5 μg of unbroken T2 DNA or 1·0 to 2·0 μg of DNA of lower molecular weight. The methods of measurement were similar to those described by Thomas & Berns (1961). Results are reported as apparent molecular weights referring to the solvated cesium salts.

3. Theory of Breakage by Stirring

When T2 DNA is stirred at various speeds under otherwise constant conditions, no change in chromatographic properties is observed up to a "critical speed" (actually a narrow range of speeds) at which breakage occurs at a measurable rate (Hershey & Burgi, 1960). If DNA is stirred continuously at the critical speed, one observes by chromatography a progressive loss of DNA eluting in the band characteristic for unbroken molecules and a progressive accumulation of DNA eluting as a single new band. The sedimentation coefficient and chromatographic properties of the DNA in the new band do not depend on the time of stirring at the critical speed. Likewise the sedimentation coefficient and the chromatographic properties of the DNA remaining in the original band are not affected by stirring.

The product obtained by stirring at the critical speed consists of an apparently continuous distribution of fragments of different lengths, as measured by chromatographic analysis and (reported below) sedimentation analysis. Any of the fragments can be broken again in a stepwise manner by stirring at the higher critical speed appropriate to its length. Therefore the original molecules are not characterized by a few preferential breakage points, either at prescribed or random locations.

These results are taken to mean that the transition from a given species of DNA to its first breakage product is a single molecular event (or a series of interrelated events). Most of the work reported in this paper is intended to show that the transition is in fact a single, central, transverse break per molecule. We begin by outlining some of the characteristics of this hypothesis.

Assume that for any given speed of stirring one can recognize molecular fragments of length L or less that cannot be broken, and fragments of slightly greater length $L + \epsilon$ that can be broken only in the region $\pm \epsilon/2$ about their centers. It follows that a given molecular structure subject to breakage under shear may be characterized by a maximum subcritical speed of stirring (defining a maximum subcritical length L_1) at which the rate of breakage is practically zero, and a faster, critical, speed (defining the critical length $L_2 + \epsilon_2 - L_1$) at which breakage occurs at some specified, measurable rate.

The same assumptions imply also that initial breaks in fragments of length much greater than $L + \epsilon$ (defined by immeasurably rapid breakage) can occur at any point between $L/2$ measured from either end.

These assumptions permit one to make certain predictions about the distribution of lengths expected under given circumstances.

(1) *Rods of identical length stirred at the critical speed.* The initial length is by definition $L + \epsilon$ and the distribution of lengths after initial breakage is symmetrical about the mean $(L + \epsilon)/2$, with a range between $L/2$ and $\epsilon + L/2$. None of the fragments produced by the initial breaks can be broken on continued stirring. This assertion implies that $\epsilon + L/2 \leqslant L$, and $\epsilon \leqslant L/2$, setting as an upper limit a twofold range of variation in lengths.

In principle the range of variation in lengths could be small, but various departures from ideality all have the effect of increasing the coefficient of variation of lengths : heterogeneity in the lengths of the starting material, heterogeneity in shear rate in different parts of the stirring vessel and particularly within microscopic regions of maximum shear, the sinuous shape of the molecules, and the practical choice of a stirring speed giving a convenient rate of breakage.

(2) *Rods stirred at greater than critical speeds.* The initial length is greater than $L + \epsilon$. All the rods are broken quickly after the start of stirring to yield a distribution of lengths that depends on time of stirring for short times, and then becomes fixed. After completion of breakage the lengths range from a seldom produced minimum of $L/2$ to a rarely surviving maximum lying between L and $L + \epsilon$. Since $\epsilon \leqslant L/2$, the range is greater than twofold and less than threefold. If the distribution is symmetrical, as seems likely, its mean is bounded by 0·75 L and L.

(3) *Ring molecules stirred at the critical speed.* The following model is pertinent to some of the results described in this paper. Imagine a closed ring of diameter about $L + \epsilon$ so that the contour length is about 3 $(L + \epsilon)$. The critical speed of stirring for such molecules might well correspond to that for linear molecules of length $L + \epsilon$. If so, after the first break opens the ring, additional breaks will occur quickly giving an apparently single step transition to perhaps 1/4 of the original molecular weight. The predicted distribution of lengths is the same as for case (2). The distribution of lengths among the fragments need not be strongly dependent on time of stirring.

The interpretation of our results according to the above principles depends, we believe, on only two assumptions concerning the hydrodynamic forces at work: that the regions of maximum shear in the stirring vessel are large compared to the lengths of DNA molecules, and that breakage at critical speeds of stirring means breakage at critical rates of shear. If the first assumption were incorrect, a molecule of DNA in a solution stirred at the critical speed could be broken into several pieces by a series of independent events. This possibility is excluded by results already mentioned and presented in detail in section 5(e) below. The second assumption calls for some discussion.

Suppose that foci of turbulence exist in the stirring vessel within which the rate of shear is much higher than it is in other parts of the solution. Suppose also that the aggregate volume of such foci forms a very small fraction of the total volume of the stirred solution. Under these conditions, DNA molecules passing through regions of maximum shear could be broken into several pieces by a single kinetic event, and the slow rate of breakage would reflect not a critical rate of shear, but the slow passage of the solution through foci of turbulence. This combination of circumstances would seem to call for highly specified conditions of stirring, whereas we find (section 5(e)) that a variety of conditions produces the same type of breakage of DNA. We therefore conclude that critical stirring speeds mean in general critical rates of shear.

4. Principle of Length Measurements

In experiments reported below, we break DNA by stirring, fractionate it into a number of length classes by chromatography, and measure the sedimentation coefficients of the fractions. We therefore ask how the distributions of lengths and sedimentation coefficients are related, and what can be learned about the actual distribution of lengths. We make the following assumptions.

(1) Before stirring, the material is homogeneous in length, and preferential breakage points, if any, are numerous and widespread.

(2) There is a normal distribution of lengths (the "number distribution") among the broken fragments, with mean $\bar{x}$ and variance σ^2.

(3) Sedimentation coefficients are related to chain lengths according to

$$\log_{10} \frac{S_2}{S_1} = k \log_{10} \frac{x_2}{x_1} \qquad (1)$$

in which S_2 and S_1 are sedimentation coefficients for molecules of lengths x_2 and x_1, respectively, and k is a constant (Doty, McGill & Rice, 1958).

(4) Resolution by chromatography is perfect so that the distributions of S and x among the fractions analysed are identical to the distributions within the material as a whole.

(5) The average length $\bar{x}$ of the distribution is known from the manner of breakage.

Within the framework of these assumptions, the experimental results can be analysed in the following way. Given a normal distribution of lengths, one passes immediately to a weight distribution (products of lengths by frequencies) given by

$$xf(x)\mathrm{d}t = \frac{x}{\bar{x}\sqrt{2\pi}}\,\mathrm{e}^{-t^2/2}\,\mathrm{d}t \tag{2}$$

in which lengths are designated by x with mean $\bar{x}$ and variance σ^2 and t is defined by

$$t = (x - \bar{x})/\sigma \tag{3}$$

The distribution (2) is nearly symmetrical even for $\sigma/\bar{x} = 1$ and has a mean (the weight average length) at $\bar{x} + \sigma^2/\bar{x}$ and a median at a slightly shorter length.

From the distribution (2) one gets, by elementary statistical calculus,

$$Z = A - \sigma f/\bar{x} \tag{4}$$

in which Z is the fractional area under the weight distribution curve to the left of some specified value of t, A is the area (given in the usual tables) to the left of the corresponding point in the normal distribution in t, and f is the frequency in the normal curve at the same point.

Equations (1), (3), and (4) form a set containing three parameters, $\bar{x}$, k, and σ, that suffice for a complete description of the weight and number distributions of x and S. In practice, one sets $x = 1$ when $S = 63$ (for unbroken DNA) and assigns some specified value (e.g., 0·5) to $\bar{x}$, and then solves the equations for the unique pair k and σ that is compatible with the measurements of Z and S for any two fractions from the column.

5. Results

(a) *Identification of half molecules*

We prepare materials for the analysis of length distributions by one of two methods, which will be described here with reference to specific examples.

A sample of T2 DNA was stirred for 30 min at 5500 rev/min and 0·45 mg/ml. and a 0·6 mg sample of the products of breakage was passed through a fractionating column. The band corresponding to broken DNA (86% of the total) eluted in 17 fractions of 3 ml. each. The band corresponding to unbroken DNA (14%) was discarded. These materials are identified below as an example of the "direct" method of analysis.

The second, or "mixed carrier" method, is illustrated by the following. A sample of ^{32}P-labeled T2 DNA was 90% broken by stirring for 30 min at 650 rev/min and $4{\cdot}5 \times 10^{-4}$ mg/ml. using the glass stirrer. To this was added 0·4 mg of carrier DNA stirred for 30 min at 5500 rev/min and 0·45 mg/ml., and 0·2 mg of carrier DNA stirred for 30 min at 7800 rev/min and 0·45 mg/ml. The mixture was then fractionated, and the band corresponding to broken, labeled DNA only was analysed. In principle, we analyse by this method fragments corresponding to the distribution of labeled DNA selected chromatographically out of an arbitrarily broader distribution of unlabeled fragments.

The direct method is more straightforward, but the mixed carrier method has several advantages: it is applicable to labeled materials in short supply; it permits one to avoid the leading edge of the band, where the resolution by our column is generally inferior (Mandell & Hershey, 1960); and it permits one to analyse DNA stirred at strictly critical speeds, which is not feasible when the direct method is used owing to the accelerating rate of breakage (caused by diminishing self-protection) that is observed when DNA is stirred at appreciable concentrations (Hershey & Burgi, 1960). The mixed carrier method requires one safeguard: one must verify that the labeled and unlabeled DNA samples are chromatographically identical after stirring in mixture. This is invariably so provided the two phage stocks have been prepared in a similar manner, and radiation damage has not accumulated.

Results of both experiments are presented in Fig. 1, together with the appropriate theoretical curve, $\bar{x} = 0{\cdot}5$, $k = 0{\cdot}55$, and $\sigma/\bar{x} = 20\%$, corresponding to the hypothesis of single breaks per original molecule of DNA. These constants locate half molecules at the 42% point of the curve, where $S = 43$. The same results were obtained in numerous checks except as noted below.

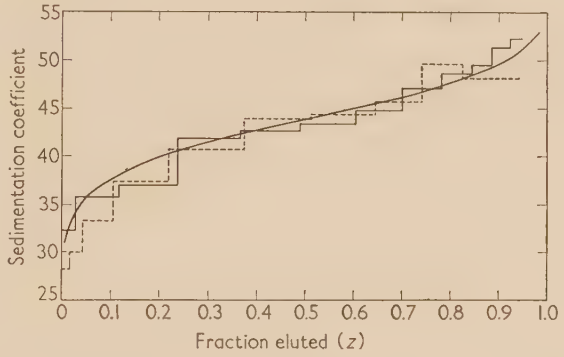

FIG. 1. Distribution of sedimentation coefficients among fragments of T2 DNA broken at its critical speed. Solid line, direct method. Broken line, mixed carrier method. Theoretical curve, $\bar{x} = 0{\cdot}50$, $k = 0{\cdot}55$, $\sigma/\bar{x} = 0{\cdot}20$.

The estimate of k, though reproducible, is arbitrary in the sense that sedimentation coefficients are arbitrary. When we measured modal instead of median sedimentation coefficients, for example, or used a plastic centerpiece for the measurements, k proved to be smaller.

The estimate of σ, though not arbitrary in the above sense, is not measurable with great precision, as Fig. 1 shows. In a first experiment of this kind we estimated $\sigma/\bar{x} = 0{\cdot}28$, although the data can be fitted almost as well by the curve shown in Fig. 1. Fortunately, considerable errors in the measurement of σ have little effect on the identification of half molecules. From the distribution (2) described earlier, it is clear that if one ignored σ and simply isolated the median fraction from the column, its molecular length would exceed that of half molecules by slightly less than the term $\sigma^2/\bar{x}$.

Several results show that imperfect resolution of lengths by our column does not seriously bias the results. (1) The successive fractions eluting from the column show a systematic trend in sedimentation coefficient (Fig. 1). (2) Only the fractions lying at the extreme left of Fig. 1 show any effect of the very different demands made on the column by the direct and mixed carrier methods of analysis. (3) Numerous tests show that the median S of the appropriate unfractionated material agrees very well with the

S measured from the median fraction from the column. (4) The range of lengths computed from k and S in Fig. 1 is about 2·3-fold. This is confirmed by an independent method described below, which shows that we are separating fairly well the longest and shortest fragments produced. (5) The fact that broken DNA isolated in a single fraction from the column, restirred at the appropriate critical speed, and again passed through a column, is resolved into two bands, shows that the DNA in the individual fractions is nearly homogeneous in length (Hershey & Burgi, 1960).

It should be added that if our column efficiently separated fragments of T2 DNA on the basis of some other variable in addition to length, the resolution with respect to length would be very poor. The arguments given above show that this complication can be excluded.

The agreement between the results of direct and mixed carrier methods of analysis shows that the distribution of lengths is about the same whether the DNA is broken at high or low concentrations, which is surprising since at high concentrations the diminishing self-protection has the effect of causing much of the breakage to occur at greater than minimum critical shear rate. We confirmed, however, that the two conditions of breakage yield chromatographically indistinguishable distributions, as shown by mixed runs with labeled and unlabeled materials. These facts indicate that DNA stirred at high concentrations is broken in its protected state: if the breakage of individual molecules occurred by escape from a protected configuration, very small fragments would be produced at the prevailing high rates of shear.

We conclude that if the materials analysed above do in fact represent the products of single transverse breaks per molecule, fragments of half the original length can be isolated near the 42% elution point of the chromatograms, and are characterized by a sedimentation coefficient of 43.

(b) *Identification of quarter molecules*

The distribution of lengths among the fragments obtained by stirring half molecules at their critical speed was analysed by the mixed carrier method as follows. Half molecules labeled with ^{32}P were isolated by chromatography as just described and the

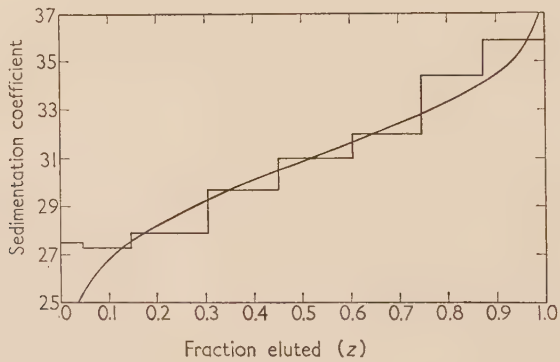

FIG. 2. Distribution of sedimentation coefficients among fragments produced by stirring half molecules at their critical speed. Theoretical curve, $\bar{x} = 0{\cdot}25$, $k = 0{\cdot}51$, $\sigma/\bar{x} = 0{\cdot}20$.

solution was stirred again (for 28 min at 0·4 μg/ml.) at a speed sufficient to break part of the DNA (Hershey & Burgi, 1960). Carrier DNA that had been separately stirred to produce an abundance of quarter length fragments was added and the mixture was

subjected to chromatographic separation. Fractions containing the labeled, broken half molecules were analysed in the centrifuge.

Results are presented in Fig. 2, in which the theoretical curve corresponds to the hypothesis $S = 43$ when $x = 0{\cdot}5$, $\bar{x} = 0{\cdot}25$, $k = 0{\cdot}51$, and $\sigma/\bar{x} = 20\%$. These parameters locate fragments of quarter length at the 42% point of the weight distribution, where $S = 30{\cdot}3$. Measurement of the fraction containing the 42% point in several other experiments also gave $S = 30$.

The distributions for fragments of half length and quarter length show the same coefficient of variation, but this measure is not extremely accurate. The difference in k is probably significant, however, and agrees with the conclusion of Doty, Marmur, Eigner & Schildkraut (1960) that k in equation (1) is not quite constant but tends to higher values as molecular weight increases. This tendency introduces a small error into our analysis of length distributions.

(c) *Viscosity measurements*

Viscosity was measured at DNA concentrations of about 10 and 20 μg/ml. in 0·2 M-NaCl, 0·015 M-sodium citrate, pH 7·4, at 25°C. Measurements were made with unstirred DNA, and with DNA stirred to produce various degrees of breakage. We did not attempt to prepare fractionated materials in sufficient quantities for these measurements. Our results show, therefore, the relation between sedimentation coefficient and viscosity of relatively inhomogeneous preparations of broken DNA. Details of some of the measurements are shown in Fig. 3. No significant effect of concentration can be seen. The

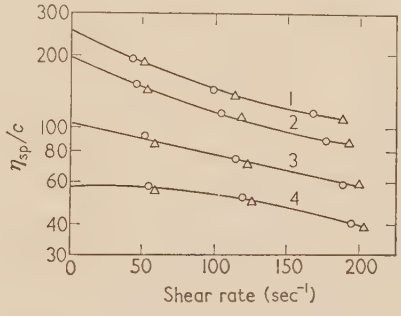

FIG. 3. Reduced specific viscosity of DNA solutions. Curve 1, unbroken DNA ($S = 63$); curve 2, $S = 45$; curve 3, $S = 28$; curve 4, $S = 21$. ○, $c = 0{\cdot}002$ g/dl.; △, $c = 0{\cdot}001$ g/dl.

reduced specific viscosity of unbroken DNA and of half molecules shows a strong dependence on shear rate at low rates of shear, as noted by Thomas & Matheson (1960). The extrapolated intrinsic viscosities for these materials are therefore dubious. The measurements were satisfactorily checked with at least two preparations of each type examined, and chromatographic checks showed that T2 DNA could be measured without breakage. Results are summarized in Table 1, together with the measured sedimentation coefficients. It will be seen that the intrinsic viscosities vary significantly with molecular weight in the high molecular weight region, though capillary viscometry is little suited to the measurements.

(d) *Sedimentation equilibrium in a density gradient of cesium chloride*

Our materials yielded band width molecular weights of about 70 million, 16 million, and 9 million, respectively, for Cs salts of whole, half, and quarter molecules identified as

already described. These estimates are obviously inconsistent with our interpretation of the breakage experiments, and the last of them is inconsistent with the molecular weight of 16 million proposed by Doty *et al.* (1960) for preparations of *E. coli* DNA that are similar to our quarter molecules with respect to sedimentation coefficient and viscosity.

These discrepancies would be simply resolved if it could be shown that breakage created heterogeneity of density in our preparations (Baldwin, 1959; Sueoka, 1959). The following experiment failed to reveal density heterogeneity.

TABLE 1

Dependence of sedimentation coefficient and viscosity on molecular weight

Relative molecular weight	Sedimentation coefficient (10^{-13} sec^{-1})	Intrinsic viscosity (dl./g)
1·0	63	250§
0·50†	43	—
0·25†	30	—
0·55‡	45	195§
0·21‡	28	105
0·11‡	21	58

† Homogeneous materials isolated chromatographically.
‡ Unfractionated DNA reduced to the indicated S by stirring. Relative weight average molecular weights interpolated from Fig. 6.
§ Doubtful extrapolations to zero shear rate.

T2 DNA labeled with ^{32}P was stirred to produce nearly complete breakage and banded in a density gradient of CsCl in a preparative centrifuge. The top and bottom edges of the band were isolated by drop collection (Weigle, Meselson & Paigen, 1959). Band width measurements of the two fractions separately and in equal mixture yielded three identical results, contrary to expectation if we had separated two fractions of unequal density. This result may mean simply that we failed to achieve adequate resolution. Brown & Martin (1955) reported marginal fractionation of T2 DNA (probably broken preparations) with respect to base composition, a result that implies density heterogeneity.

On the basis of arguments presented below, we believe that some or all of our band width molecular weights are spurious, but cannot account for the discrepancies.

(e) *Evidence for a single-break mechanism*

If the relative band width molecular weights cited above were to be accepted, one should conclude that the hypothesis of single, centrally located breaks is correct for the production of $S = 30$ fragments from $S = 43$ fragments, but incorrect for the production of $S = 43$ fragments from our starting material ($S = 63$). The latter would require at least three breaks per molecule. Specifically, one could imagine that for very long molecules, susceptibility to breakage is little dependent on length, so that initial breaks are not restricted to the central region, and multiple breaks ensue at the critical rate of shear. What are some consequences of this hypothesis?

Qualitatively, one predicts that if quarter length fragments can be produced, so can three-quarter lengths, and the latter should show about the same susceptibility to breakage as the starting material. Thus as stirring is continued, an initially broad distribution of lengths should gradually become narrower concomitantly with a decrease in the average length.

According to the stated hypothesis, the observed distribution can be analysed as follows. From the band width measurements, we are given $x = 1$ when $S = 63$, $x = 0{\cdot}25$ when $S = 43$, and $x = 0{\cdot}125$ when $S = 30$, approximately. We may assume that a plot of log S against log x permits interpolation between these points of reference. Then, from the observed distribution of S in Fig. 1 (solid line), and without further assumptions, we arrive at a number distribution of lengths characterized by $\bar{x} = 0{\cdot}25$, $\sigma/\bar{x} = 0{\cdot}29$, and a 3·2-fold range. This range of variation is excessive according to the theory of multiple breakage outlined earlier, which could mean that breakage had not proceeded to equilibrium, that the breakage theory is inappropriate to the hydrodynamic situation prevailing in the stirring vessel, or that the assumption $\bar{x} = 0{\cdot}25$ is incorrect.

TABLE 2

Time-independence of the size distribution of DNA broken at its critical speed

Stirring conditions			Percentage broken	Median elution point	Median S
stirrer	rev/min	min			
steel	310	30	10	0·75	—
steel	310	30	24	0·74	43·7
glass	610	30	34	0·71	44·8
glass	650	30	42	0·73	44·2
glass	640	60	81	0·72	43·3
glass	660	45	100	—	43·2
glass	640	240	100	0·66	42·2
steel	320	240	100	0·72	43·9

50 ml. of ^{32}P-labeled DNA (0·4 μg/ml.) was stirred as stated and fractionated chromatographically in mixture with a heterogeneous preparation of broken carrier DNA. From the chromatogram the percentage of labeled DNA broken, and the fraction of carrier DNA eluting in advance of the median point in the band corresponding to broken, labeled DNA, was measured. Sedimentation coefficients were measured for three fractions bracketing this median elution point, and the results were interpolated to that point.

Table 2 summarizes some measurements in which the median length and median S of DNA broken by stirring at the critical speed is related to time of stirring. With the possible exception of one of the samples stirred for 4 hours, no effect of stirring time can be detected. Fig. 4 displays the length distributions for two of the samples as reflected in chromatographic comparisons. Again there is no sign of progressive breakage. Evidently the distribution is nearly or wholly independent of time of stirring from times so short that only 10% of the DNA is broken to times considerably longer than that required for complete breakage, a result contrary to the expectations for a multiple break process, and excluding insufficient stirring time as a determinant of the distribution.

Table 2 and Fig. 4 also show that two stirrers whose gross action is very different produce the same distribution of lengths. As mentioned earlier, the identical distribution

is likewise produced by a high speed cutting blade turning at the critical speeds appropriate to more concentrated solutions of DNA. These results show that the observed distribution does not reflect a unique hydrodynamic situation in the stirring vessel.

A somewhat different test of the hypothesis of multiple breaks was performed by isolating chromatographically the longest fragments resulting from the breakage of labeled DNA, and testing their susceptibility to breakage under the conditions in which they had been produced. For this purpose the labeled fragments were mixed with

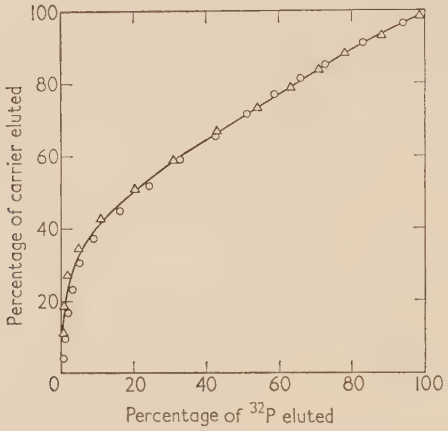

FIG. 4. Chromatographic comparison of labeled DNA samples broken partly and completely at the critical shear. ⊙, DNA 34% broken after stirring for 30 min (glass stirrer, 610 rev/min). △, DNA completely broken after stirring for 4 hr (steel stirrer, 320 rev/min). Each sample was separately mixed with stirred carrier DNA and passed through a column.

unbroken carrier DNA, and the mixture was stirred (at 0·15 mg/ml.) so as to break about 80% of the carrier DNA in 30 min. The mixture was then passed through a column to compare the distributions of labeled and unlabeled DNA. Tests of this kind showed that fractions eluting to the left of the 90% point of the original chromatogram ($S \leqslant 50$) showed negligible breakage on restirring. Fractions eluting to the right of the 90% point of the chromatogram (S not measurable because of low DNA concentrations) showed on restirring small but significant degrees of breakage, which increased progressively with each succeeding fraction. One example is reproduced in Fig. 5, which refers to the 96 to 98% fraction of the original chromatogram. The figure shows that 36% of the labeled DNA has been broken, 23% by the stirring, and (as revealed by an unstirred control) 13% spontaneously during the fractionation and collection of the DNA, which in this fraction emerged from the column at a concentration of 1 μg/ml. Figure 5 also shows that on breakage the labelled DNA moves from the extreme right to the extreme left of the distribution corresponding to broken carrier. If the evidence for a single break process cited above is accepted, the result illustrated in Fig. 5 can only mean that the breakage point of the labeled fragments is central, and that the range of the length distribution of unlabeled fragments is about twofold.

The above experiment confirms the less sensitive tests of Table 2 in showing that the small number of the largest fragments found in stirred DNA is due primarily to their infrequent production, and only to a small extent to their subsequent breakage.

The restirring experiments just described also measure the unbreakable length L characteristic of the critical speed of stirring actually used. It corresponds to $S = 50$,

or $x = 0{\cdot}66$ according to the theory of Fig. 1. Since the unit length is by definition $L + \epsilon$ for the same conditions of stirring, $\epsilon = 0{\cdot}34$, or about $L/2$. Thus the rapidly breakable length is about 1·5 times the unbreakable length, in agreement with the theoretical prediction for a time-independent distribution with a twofold range. The agreement is consistent with our assumption that potential breakage points are numerous and uniformly distributed along the molecular lengths.

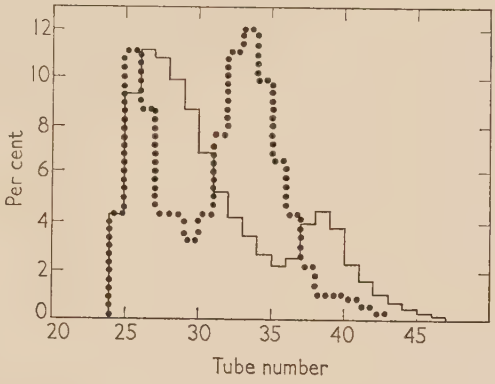

FIG. 5. Simultaneous breakage of native DNA and labeled long fragments. A sample of ^{32}P-labeled DNA was stirred to produce nearly complete breakage, and separated chromatographically into fractions of different molecular length. Samples of these fractions were mixed with unstirred carrier DNA, and the mixture was stirred again to break most of the carrier and passed through a second column. The figure shows the result for the 96 to 98% fraction ($S > 50$) of the labeled, broken DNA. Solid line, carrier DNA (80% broken); dotted line, labeled DNA, 36% broken. A control showed that 13% of the labeled DNA was broken before stirring.

The arguments presented above are diverse and may be summarized as follows. (1) The time-independence of the length distribution resulting from breakage at critical rates of shear shows that (except rarely) only one break is produced per molecule, or at least that breakage is not characterized by a succession of independent events per molecule. (2) This conclusion is also supported by the finding that the distribution is the same for three very different conditions of stirring, since a multiple break process should depend on the size, number, and mobility of microscopic regions of maximum shear in the stirring vessel. (3) The products of breakage form a single chromatographic band well separated from the unbroken molecules, which means that the breaks must be centered with respect to molecular lengths. (4) When the longest fragments produced by initial breaks are broken in turn, the modal length of the resulting fragments is similar to the length of the shortest fragments produced by initial breaks. The range of the length distribution of fragments resulting from initial breaks is therefore about twofold.

We conclude that the breakage theory presented earlier in this paper is applicable to our experiments, and that the hypothesis of multiple breakage scrutinized in the preceding paragraphs is incorrect.

(f) *An alternative interpretation of the breakage process*

The foregoing disproof of the hypothesis of multiple breakage is not quite general because it makes use of an unproved assumption: that sedimentation coefficients are related to chain lengths by a continuous function that serves both for broken and unbroken DNA. One might suppose, on the contrary, that the two kinds of DNA are

structurally nonhomologous. The ring model presented earlier, for example, explains how a molecule of DNA subjected to critical shear could be broken into quarters by an apparently single step process. At the same time the sedimentation coefficient of the unbroken DNA could not be used to predict the sedimentation coefficients of the breakage products, whereupon our argument that the presumed quarter-length distribution shows an excessive range of variation disappears. To obtain a reasonable range of variation with $\bar{x} = 0{\cdot}25$ and the data of Fig. 1, it is sufficient to assume that relation (1) holds with $k = 0{\cdot}5$, from which it follows that T2 DNA would show a sedimentation coefficient of about 86 if it were not for the hypothetical structural singularity. (The consistency of this assumption with the band width measurements could be tested by extending them to all the fractions in Fig. 1.)

Two arguments can be adduced in opposition to this hypothesis. First, T2 DNA and its longer breakage products form a nearly continuous series with respect to chromatographic properties, as would be predicted from their sedimentation coefficients. Second, when critical stirring speed is measured in relation to sedimentation coefficient for T2 DNA and its breakage products, a continuous function is revealed (unpublished experiments).

To sum up, T2 DNA could have a molecular weight about four times that of its first breakage products, as suggested by the band width measurements, only provided that the unbroken molecules were structurally different from the fragments resulting from breakage. Taking the latter as a norm, the structural singularity would have to be such as to cause the molecule to break into quarters as a single event when subjected to critical rates of shear; to reduce the sedimentation coefficient from about 86 to 63; to lower the affinity for basic protein to about what should be expected for uncomplicated molecules of $S = 63$; and to increase the critical shear rate to about what should be expected for uncomplicated molecules of $S = 63$. If the band width molecular weights should be confirmed by another method, these coincidences would have to be taken seriously. In the meantime we conclude that the band width measurement is not applicable to our materials, and that we have correctly identified half molecules at $S = 43$, and quarter molecules at $S = 30$.

6. Discussion

Perhaps the most remarkable feature of our results is qualitative: we find that T2 DNA and its breakage products form a nearly continuous series whose members fall into a single order whether classified by chromatographic behaviour, sedimentation coefficient, viscosity, or susceptibility to further breakage by hydrodynamic shear. We conclude that we are dealing with a single variable, molecular length, within a structurally homologous series. T2 DNA consists, therefore, of linearly extended chains with numerous, uniformly distributed breakage points of equal fragility.

A second remarkable feature of our results is an exception to the first: we are unable to prepare fragments of T2 DNA having a sedimentation coefficient between 63 (characteristic of unbroken DNA) and about 53. To some extent this fact weakens our first conclusion. A sufficient explanation is found, however, in our hypothesis, rather completely verified in this paper, that molecules of T2 DNA are of uniform length and, under critical shear, can be broken only in the central third of that length, owing to the way in which hydrodynamic forces operate. Making use of this principle, we have isolated homogeneous preparations of half-length fragments ($S = 43$) and quarter-length

fragments ($S = 30$). The molecular weights of these materials are unknown and, specifically, measurement by equilibrium sedimentation in a density gradient is not applicable to them.

The physical measurements for T2 DNA (Table 1) are in reasonable agreement with those of Davison (1959) and Thomas & Berns (1961). Other published descriptions refer to preparations that, by our criteria, were broken (Thomas & Matheson, 1960; Fleischman, 1960).

Judging by sedimentation coefficient and viscosity, T2 DNA fragments of the quarter length have a slightly lower molecular weight than that of a preparation of *E. coli* DNA (Doty *et al.*, 1960). The mutual consistency of the two types of measurement encourages one to combine data from diverse sources as shown in Fig. 6. The molecular weights for

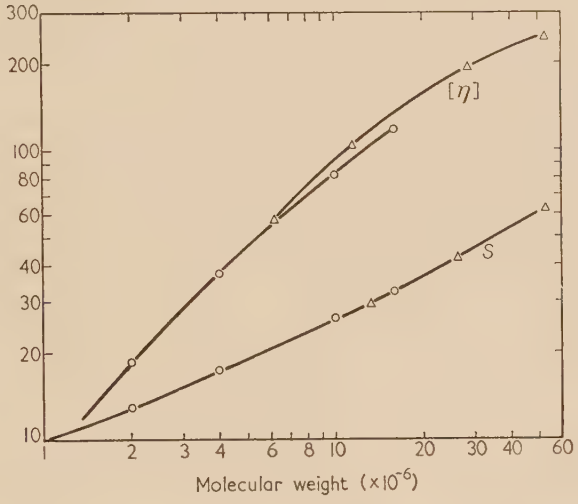

FIG. 6. Provisional relations between molecular weight and physical properties of diverse DNAs. ○, the series of Doty *et al.*, 1960. △, the T2 series with a molecular weight of 53 million arbitrarily assigned to unbroken DNA.

our materials arrived at in this way depend directly on the estimate of 16 million (itself the result of extrapolation) assigned to *E. coli* DNA. If the latter is correct, the DNA of T2 has a molecular weight of 53 million.

A molecular weight of 45 million was computed by Levinthal & Thomas (1957) from the rate of disintegration of incorporated radiophosphorus measured autoradiographically. Their preparations were inhomogeneous and apparently broken, however, and the method requires further study (Thomas & Berns, 1961). The latter authors suggest a molecular weight of 60 million based on density gradient equilibrium measurements. In our opinion these various estimates agree in showing that the molecular weight is high, but do not exclude, for instance, a value of about 120 million expected if the phage particle contains a single molecule of DNA. We suggest as a minimum requirement for future measurements that they yield consistent results with our fourfold series.

The methods outlined in this paper require as starting material DNA that is homogeneous both with respect to molecular weight and with respect to other properties that may affect chromatographic behaviour. Their applicability, therefore, is likely to be limited. We are investigating the possibility of an extension of the methods that may prove more generally useful.

We wish to thank Dr. C. A. Thomas, Jr. and Dr. A. T. Matheson for assistance with the construction of a viscometer and instruction in its use; Dr. H. K. Schachman for advice concerning measurement of sedimentation coefficients; Professor Paul Doty and Dr. Joseph Eigner for the gift of a sample of *E. coli* DNA; and Professor C. Levinthal and Dr. P. F. Davison for discussions of their theory of the hydrodynamics of breakage.

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Letters to the Editor

Remark concerning the Rate of Uncoiling of Helices of Two or More Strands

The problem concerning the time requirement for a longitudinal fission by Brownian movement of a very long particle consisting of two (or more) filaments twisted a great number of times round each other to form a double (or multiple) spiral was discussed a few years ago (Kuhn, 1957). It was shown that a comparatively swift disintegration is obtained by partial rotational or torsional *Brownian movement* round the axis of the spiral† resulting in a loosening of the spiral structure and subsequent separation of the constituents by translational Brownian movement. The time required to separate a Watson-Crick double spiral (Watson & Crick, 1953*a*,*b*) of DNA consisting of about 900 turns of height of $3{\cdot}4\times10^{-7}$ cm each and a radius of 10^{-7} cm, thus having a length of 3×10^{-4} cm, was found by this mechanism to be about 50 to 80 sec. In an appendix of the same paper a consideration was advanced, according to which the time required to undo the same spiral by unwrapping it turn by turn by Brownian movement of the free ends of the chain would be about 150 days.

This problem has been rediscussed in a paper entitled "Calculation of the Rate of Uncoiling of the DNA Molecule" (Longuet-Higgins & Zimm, 1960). Two remarks are made relative to it.

1. The paper of Longuet-Higgins & Zimm contains some criticism concerning the *appendix* of my 1957 paper, without mentioning the result obtained in the main paper, according to which rotatory Brownian motion of the molecular axis in itself is capable of achieving an essential uncoiling within *one second* and a complete separation of the strands within 50 to 80 sec, without any other agent than the completely "blind" Brownian movement. This omission is regrettable because this mechanism will and must be working independent of, and possibly beside, other mechanisms which might be operating.

2. When trying to understand the alternative mechanism of a turn-by-turn unwinding of the coil, and tentatively accepting the interesting idea of a small torque originating for entropy reasons near the axis of the coil at the point where the free ends unite in the partly untwisted coil, it is difficult to see how such a torque would be transmitted through the statistically shaped unrigid filamentous parts over a distance for example of 100 Å to the center of gravity of the statistical coil formed by the free ends in order to make these ends move in the right direction. That is to say I see a difficulty in transmitting a momentum by means of a soft non-rigid flexible connection.

† The case of a rotational movement round the axis of the spiral under the influence of a driving force (but not under the influence of Brownian movement) was previously discussed by Levinthal & Crane (1956).

A more careful consideration of this point leads back to the importance of a spinning Brownian movement of the spiral axis and thus to the essential feature of the earlier consideration (Kuhn, 1957).

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Further Remarks concerning the Rate of Uncoiling of Helices of Two or More Strands

We regret that in our preoccupation with the discussion of our own proposal we should have unfairly omitted reference to the main part of Professor Kuhn's paper (1957). In our view, the relation between the two papers is the following: we believe that the mechanism of uncoiling that he has proposed may be a fairly close approximation to the real molecular process, but we feel that the idea of a decrease of free energy driving the uncoiling should also be introduced. However, the computations appear to be difficult when our idea is applied directly to Professor Kuhn's mechanism. For this reason we were led to carry through the calculations for a simpler process, that of turn-by-turn uncoiling from the ends. The actual result for the total rate of uncoiling, which is the sum of the rate of uncoiling in the middle and of the rate of uncoiling from the ends, should not depend very much on the details of the process assumed for the calculation, because of the fact that both kinds of uncoiling are coupled to the same resistance.

In the particular model that we assumed the free energy decreased strictly linearly with the amount of uncoiling. Under this assumption the uncoiling should proceed from the ends only, for reasons discussed in more detail in our paper. In a more realistic model the free energy decrease might not be strictly linear, and uncoiling in the middle would also be allowed. However, the total rate should be nearly the same.

In regard to Professor Kuhn's second point, our idea is that the torque would be transmitted from the axis to the rest of the random coil by the same forces that give rise to rubbery elasticity, i.e. the tendency of the entropy of the coil to reach a maximum, It is true that the application of the torque would distort the random coil by twisting it. and that this distortion would affect the value of the hydrodynamic resistance to rotation of the coil. How serious this effect would be we do not know, but the similar effect of distortion on the shear viscosity of polymer coils in dilute solution is slight, less than a factor of two.

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Effect of Urea and Heat Treatments on the Activity of Soluble Ribonucleic Acid

Ribonucleic acid present in the soluble fraction of cell homogenates (S-RNA) plays an important role in protein synthesis: it accepts amino acids at its chain end and transfers them to ribosomes (Hoagland, 1955; Hoagland, Zamecnik & Stephenson, 1957; Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958). Recently, evidence for a secondary structure was found in the S-RNA prepared from *Escherichia coli* (Tissières, 1959; Cox & Littauer, 1960); *E. coli* S-RNA manifests a hyperchromic effect, similar to that observed with the RNA of tobacco mosaic virus and of ribosomes (Hall & Doty, 1958; Doty, Boedtker, Fresco, Haselkorn & Litt, 1959). In an attempt to investigate the reversibility of "denaturation" of S-RNA with respect to its biochemical activity, the effect of urea or heat treatment has been examined in the work to be reported here.

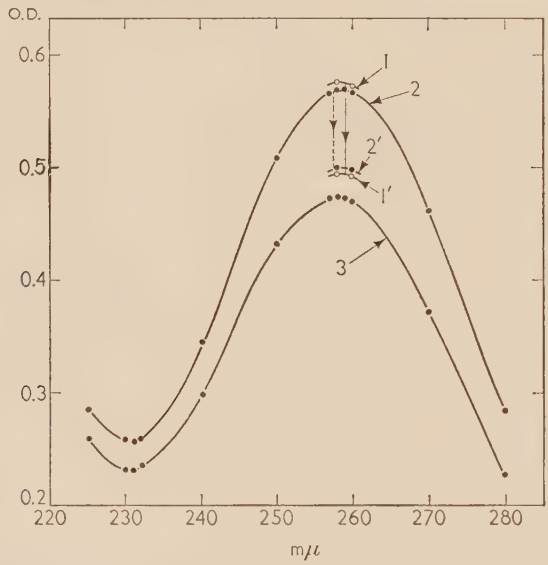

FIG. 1. Changes of ultraviolet absorption spectrum of S-RNA on treatment with heat or urea (in 0·1 M-phosphate buffer, pH 7·0).

1—at 83°C; 1′—cooled to 18°C;
2—in 6 M-urea; 2′—after dialysis;
3—untreated control.

S-RNA was prepared from the pH 5 fraction of rat liver homogenate by the method of Hoagland *et al.* (1958). The molecular weight of this S-RNA, calculated from its sedimentation coefficient ($S_{20,w} = 4{\cdot}1$s) and diffusion coefficient ($D_{20} = 8{\cdot}9 \times 10^{-7}$ cm^2/sec), was 27,000, assuming that the partial specific volume was 0·58 (Cohen & Stanley, 1942). The S-RNA was further purified by chromatography on DEAE-cellulose (Cl^-) (Nishiyama, Okamoto, Watanabe & Takanami, 1961) using, however, NaCl and acetate buffer (pH 5·5) as eluent instead of LiCl and Li-acetate (pH 4·9). In the experiments to be described, S-RNA was eluted from the column in the range 0·4 to 0·7 M-NaCl.

In order to measure the hyperchromic effect of the S-RNA preparation, the S-RNA was hydrolysed with 0·3 N-KOH for 20 hr at 37°C yielding a mixture of 2′ and 3′ nucleotides. This hydrolysis produced an increase in optical density at 260 mμ, or total hyperchromic effect, of 49%. The contribution of secondary structure to this hyperchromic effect was estimated by heating a solution of S-RNA in 0·1 M-phosphate buffer (pH 7·0) at 80°C for 10 min, or by treatment with 6 M-urea for 30 min at room temperature. Both heating and urea treatment produced an increase of optical density at 260 mμ of about 21% (Fig. 1). The increase in optical density observed on heating and urea treatment were reversed 80% and 71% upon cooling or dialysis, respectively.

The effect of heat and urea treatments on S-RNA in relation to the incorporation of amino acids

Rapidly cooled S-RNA solution after heating at 80°C for 10 min, and dialysed S-RNA solution after 6 M-urea treatment for 30 min at room temperature, were precipitated by

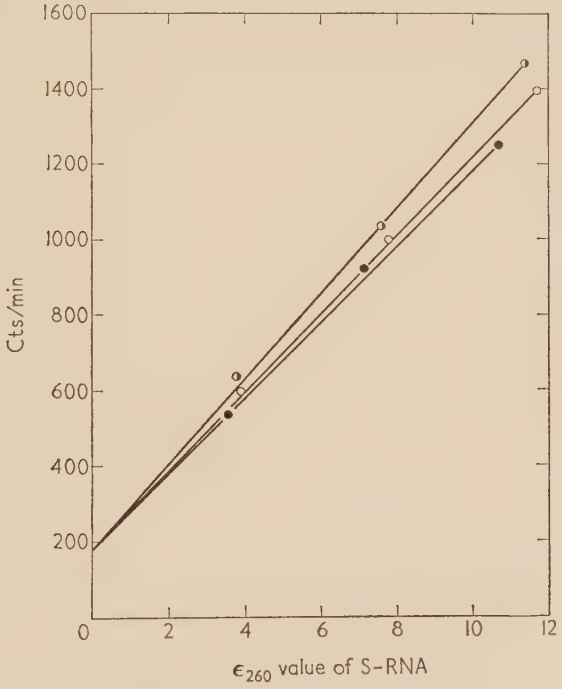

Fig. 2. Amino acid incorporation into S-RNA. The incubation mixture contained, in a final volume of 4 ml.: 0·2 μmole (2×10^5 cts/min) [^{14}C]leucine; 20 μmoles ATP; 3·7 mg enzyme fraction; 20 μmoles $MgCl_2$; 120 μmoles KCl; 200 μmoles tris, pH 7·6; and a varying concentration of S-RNA. Incubation was 15 min at 37°C. ^{14}C-activity was counted by a low background gas-flow counter.

●——● not treated;
○——○ treated with 6 M-urea, 30 min;
◑——◑ heated at 80°C for 10 min.

adding 2 vol cold ethanol, dissolved to a concentration of 2 mg/ml. and dialysed against distilled water. The S-RNA solutions were then incubated with L-[^{14}C]leucine (uniformly labeled, from the Radiochemical Centre, England), ATP, $MgCl_2$, KCl and an enzyme

fraction prepared from the pH 5 enzyme fraction in tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol) pH 7·6. After incubation for 15 min, the reaction mixture was cooled in an ice bath and S-RNA was extracted from the reaction mixture by the phenol method (Kirby, 1956). The extract was twice precipitated with cold ethanol, twice washed with cold 0·2 N-perchloric acid (PCA), and the ^{14}C-activity of the precipitate was counted. Figure 2 shows the amount of radioactive amino acids incorporated into S-RNA that has been subjected to heat or urea treatment. These results clearly show that neither heating nor urea treatment of S-RNA destroys its ability to incorporate amino acid.

The effect of heat and urea treatments on S-RNA in relation to amino acid transfer from S-RNA to ribosome

S-RNA was labeled with [^{14}C]leucine by the method of Hoagland *et al.* (1958) and further purified by chromatography on DEAE-cellulose. The labeled S-RNA amino acid complex thus prepared was treated either by heating or by incubation with 6 M-urea in exactly the same way as described in the previous section. These pre-treated S-RNA-amino acid complexes were incubated with ribosomes, the transfer enzyme, and other cofactors, in order to measure the transfer of the amino acid to ribosomes. After incubation for 30 min at 37 °C, the reaction was stopped by cold 0·5 N-PCA and the ^{14}C-activity transferred to ribosomes was determined (Takanami & Okamoto, 1960).

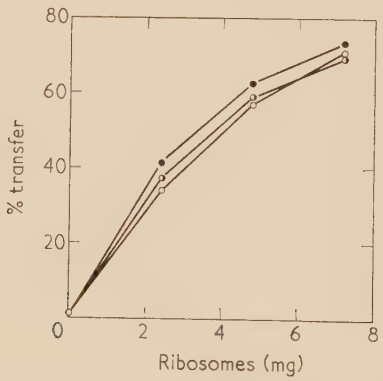

FIG. 3. Amino acid transfer from S-RNA to ribosome. Incubation mixture contained, in a final volume of 2 ml. transfer enzyme: 0·7 mg, GTP; 0·6 μmole, $MgCl_2$; 10 μmoles, tris pH 7·6; 100 μmoles, S-RNA [^{14}C]leucine and a varying concentration of ribosome. Incubation time was 30 min at 37 °C.

●——● not treated (380 cts/min, $\epsilon_{260} = 3{\cdot}0$);
○——○ treated with 6 M-urea 30 min (316 cts/min, $\epsilon_{260} = 3{\cdot}1$);
◑——◑ heated at 80 °C 10 min (245 cts/min, $\epsilon_{260} = 3{\cdot}4$).

Efficiency of transfer was expressed as the percentage of the radioactivity transferred from S-RNA to ribosomes during the incubation period. Figure 3 shows the results of this experiment. It can be seen that neither heating nor urea treatment of the S-RNA has much effect on its ability to transfer amino acids to ribosomes. In this connection, it should be added that the ability of ribosomes to accept amino acids from S-RNA is highly sensitive to temperature and hydrogen bond breaking agents (to be published).

Conclusion

From the experiments described above it follows that urea or heat treatment which causes reversible physical changes in S-RNA does not destroy irreversibly the known biochemical activity of S-RNA. Thus it seems that the activity of S-RNA depends on the integrity of the RNA molecule, but not on a specific physical superstructure. However, the possibility of the existence of an essential physical structure which is reversibly altered by the treatments used has not been excluded.

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On the 1700 cm^{-1} Band of the Cross-β Structure

In a recent issue of this journal, Bradbury *et al.* (1960) reported their finding of a "cross-β" structure in some synthetic polypeptides. Quite independently, we had found essentially the same structure in films of low molecular weight poly-γ-benzyl-glutamate. For this particular polypeptide, however, the infrared absorption curve we obtained is somewhat different from that of Bradbury *et al.* Moreover, we have obtained additional information by studying the weak band at 1700 cm^{-1}.

Samples of either poly-γ-benzyl-L-glutamate or poly-γ-benzyl-D-glutamate of mean degree of polymerization (D.P.) 8 were studied as 1% solution in ethylene dichloride. Just after being prepared, such solutions were not highly viscous and no oriented films were obtainable from them. After a week or so, however, the

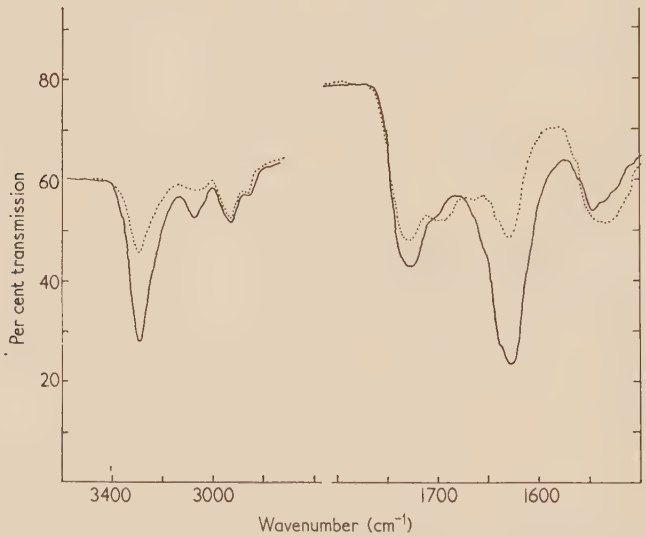

FIG. 1. Infrared absorption spectrum of oriented poly-γ-benzyl-D-glutamate (mean degree of polymerization 8). Full line, E-vector parallel to fiber axis; broken line, E-vector perpendicular to fiber axis.

solution became very viscous, so that it was possible to spin it into fibers; it also exhibited optical anisotropy, forming liquid crystals. An oriented film was obtained by spreading the solution on a potassium chloride plate; after the solvent had evaporated, the residue was rubbed in one direction with a spatula. The film then exhibited strong positive birefringence, and its infrared absorptions are shown in Fig. 1. The parallel dichroism of the 1630 cm^{-1} (amide I of a β-structure) and 3300 cm^{-1} (NH stretching) bands indicate that this film has a structure in which extended (or nearly extended) polypeptide chains are arranged parallel (or antiparallel) to one another and perpendicular to the direction of rubbing (fiber axis) . . . namely, a "cross-β" structure.

From a sample of poly-γ-benzyl-L-glutamate of approximate D.P. 20, Bradbury *et al.* (1960) obtained an oriented film the infrared spectrum of which indicated that

"cross-β" and "α-helix" structures were present together. Our sample of mean D.P. 8 gives a pure "cross-β" structure. It would thus appear that to obtain a pure "cross-β" structure of poly-γ-benzyl-L-(or D-)glutamate a D.P. lower than 20 is necessary. This is of interest in connection with the crystal structures of much smaller "polypeptides." Thus, we have found a similar "cross-β" structure in the crystal of acetyl leucine N-methylamide and in some other acetylamino acid N-methylamides. Acetyl leucine N-methylamide, for example, forms crystals of needle-like habit, and its NH stretching band (3290 cm^{-1}) and C=O stretching band (at 1616 cm^{-1}) are polarized parallel to the direction of elongation of the crystal (Mizushima *et al.*, 1953).

A film with the "cross-β" structure has also been obtained from an ethylene dichloride solution in which poly-γ-benzyl-L-glutamate and poly-γ-benzyl-D-glutamate were dissolved in equal proportions. The infrared absorptions of this film are shown in Fig. 2. The 1630 cm^{-1} and 3300 cm^{-1} bands, both polarized parallel to the fiber

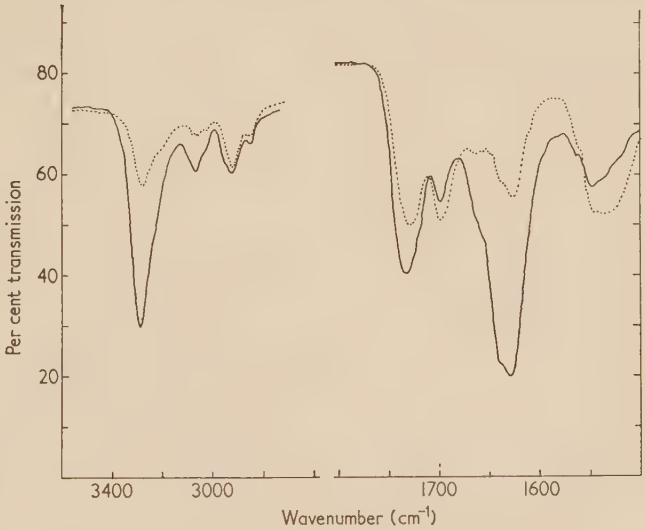

FIG. 2. Infrared absorption spectrum of oriented film composed of poly-γ-benzyl-L-glutamate and poly-γ-benzyll-D-glutamate in equal proportions. Full line, E-vector parallel to fiber axis; broken line, E-vector perpendicular to fiber axis.

axis, are similar to those of the pure L- or D-film. However, there is an appreciable difference in the relative intensity of a band at 1700 cm^{-1}, which is stronger for the D and L mixture film than for the pure L- or D-film. From its position, intensity and dichroism, this band is assignable to one of the two amide I bands split from one band by a vibrational interaction between two fully or nearly fully extended polypeptide chains arranged antiparallel to each other. An analysis of such an interchain interaction has been made by Miyazawa (1960), according to which the intensity of this band should be approximately proportional to $\cos^2\phi$, where ϕ is the angle between the transition moment direction of the isolated peptide group and the chain axis. We may consider, therefore, that ϕ is smaller in the mixed D and L film than in the pure L- or D-film, and therefore that the polypeptide chains are more fully extended in the former.

From the results just quoted, it may be concluded that the "cross-β" structure composed wholly of D- or of L-polypeptide chains is that of an "antiparallel chain

pleated sheet" described by Pauling & Corey (1953). These authors have also proposed another model in which the structure is composed of D-chains alternating with L-chains, named the "antiparallel chain rippled sheet." This configuration, however, does not account for our observation of the intensity of the 1700 cm^{-1} band. On the basis of this and some other evidence, we consider an "antiparallel chain flat sheet" to be present in the "cross-β" structure composed of D- and L-chains. Details of this model will be published shortly.

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INSTRUCTIONS TO AUTHORS

1. The *Journal of Molecular Biology* will publish papers on the nature, production and replication of biological structure at the molecular level, and its relation to function. Suitable subjects are sub-cellular organization: molecular genetics: structure and replication of viruses: molecular structure of muscle, nerve and other tissues: structure of proteins, nucleic acids, carbohydrates, lipids, etc., and their synthetic analogues, as investigated by X-rays, light absorption and other methods: problems of inter- and intra-molecular energy transfer. The Journal will not as a rule publish papers when the subject matter is outside the fields defined above; however, when the subject matter borders on molecular biology and is of special interest in relation to molecular biology such papers may be published when space permits.

2. The Journal will also publish Letters to the Editor (averaging 1000 words), and occasional review articles dealing with a more restricted or immediately topical field than those in review journals. (The Editor should be consulted in advance by anyone who considers submitting such a review.)

3. Communications will often describe results obtained by specialized techniques (for example, those of X-ray crystallography and of genetics), and it is most important that technical terms should be avoided or be clearly defined; in any event the actual results obtained and their implications should be described in a way which does not demand from the reader specialized knowledge of a particular technique.

Papers should contain the maximum density of information compatible with clarity, and every sentence should be scrutinized to ensure that its presence is essential. Authors should include enough experimental details to enable others to repeat the work (these may be printed in small type). Careful preparation of manuscript will aid prompt publication; carelessness will cause delay.

4. *Submission of Manuscripts.* Manuscripts should be sent to Dr. J. C. Kendrew, Peterhouse, Cambridge, England, or to any member of the Editorial Board. The submission of manuscript will be taken to imply that the material is original, and that no similar paper has been or will be submitted for publication elsewhere.

Papers will normally be published in English. Manuscripts in French or German will also be accepted, though the use of these languages may cause delay in publication.

5. *Copy.* Papers should be typewritten in double spacing *throughout* (including tables, legends and footnotes), on one side of quarto paper, with a margin of 1 inch all round. The position of tables and illustrations should be indicated in the text; tables and legends for illustrations should be typed separately at the end of the manuscript. All pages should be numbered serially and securely fastened together. Two copies of each manuscript should be submitted, together with three copies of the summary (see § 6) and four copies of the abstract (see § 9).

A short running title of not more than 40 characters (including spaces), suitable for page headings, should be indicated if the full title is longer than this.

The name of the laboratory where the work has been done should be indicated on the title page. All typescript (including the references) should be carefully checked for errors.

6. *Summary.* A summary will be printed at the head of all papers (excluding Letters to the Editor); this should generally not exceed 300 words, and should be intelligible to the general reader without reference to the main text. Abbreviations should be avoided. Summaries of papers to be printed in French or German will be in English, and should be longer (up to 1000 words) for the benefit of readers not familiar with these languages.

7. *Conventions.* The conventions used in current issues of the Journal for headings, references, etc., should be followed in preparing manuscripts. References should be listed alphabetically at the end of the paper, and abbreviated according to the *World List of Scientific Periodicals*, except that for periodicals with English names capital initial letters should be used throughout. Citations in the text should read thus: Smith, Brown & Robinson (1957), or (Smith, Brown & Robinson, 1957). When a citation has more than two authors, the names of all of them should be given at the first citation (except for more than five authors), and on subsequent citations thus: (Smith *et al.*, 1957). The conventions (Brown, 1957*a*), (Brown, 1957*b*) should be used where more than one paper by the same author(s) has appeared in one year. Volume number should be indicated in the typescript thus: **35**.

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American Society for Cell Biology

The newly formed AMERICAN SOCIETY FOR CELL BIOLOGY has announced its first regular meeting and has issued a call for papers. The meeting will be held November 2, 3, and 4, 1961, in Chicago, Illinois at the Edgewater Beach Hotel. A program of three symposia, contributed papers, demonstrations and motion pictures is being arranged. The symposia, with invited speakers from the U.S. and abroad, reflect the Society's aim to transcend disciplinary boundaries and bring together workers with divergent approaches in an effort to focus on fundamental problems of the cell. The topics will cover the following:

I. Cell continuity: The molecular, ultrastructural and genetic aspects of replicating systems of the nucleus and cytoplasm.

II. Cell diversification: Clonal diversity in cell structures and aspects of differentiation in plant and animal cells.

III. Characteristics of cell interfaces: The chemistry and the fine structure of cell membrane systems, and problems of transport across membranes.

All interested cell biologists, whether they are members or not, are invited to submit papers (15 minute presentation time including discussion) for the contributed papers sessions. Demonstration papers and motion pictures are also solicited. The Program Committee includes the following:

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Further information about the Society or the meeting may be obtained from the Acting Secretary, DR. M. J. MOSES, Box 2982, Duke University School of Medicine, Durham, North Carolina.

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